The Development of Non-Invasive Genetic Methods for Bats of the British Isles

A thesis submitted to

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by

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Under the supervision of Dr. Catherine O'Reilly and Dr. Peter Turner

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Declaration

The work described in this thesis is original and was solely carried out by the author, and the work of others has been duly referenced in the text. No part of this thesis has been previously submitted for a degree at this or any other institute.

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Andrew P. Harrington

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Abstract

Bats play an important role in the ecosystem of the British Isles, but are vulnerable to population decline due to human activities and thus are the subject of much scientific research. Non-invasive DNA sampling is commonly used in scientific studies of wild mammal species, but is still used in relatively few genetic studies of bat species. The overall aim of this thesis is to develop molecular techniques for application to the bat species of the British Isles.

Species-specific real-time PCR primers, targeting the cytochrome b mitochondrial DNA gene, were designed for the identification of eighteen resident bat species in the British Isles. These primers were applied to a field survey of bat roosts in Ireland, in Counties Galway, Kildare, Waterford and Wexford, from which non-invasively collected faecal DNA samples were obtained.

The lesser horseshoe bat (*Rhinolophus hipposideros*) is one of Ireland's rarest bat species, and its population is monitored nationally each summer by emergence counts of at known summer roosts. The sex ratio of adult bats present in these roosts is an important part of calculating national population estimates, but no empirical data on this sex ratio are available from Ireland. Real-time PCR sex typing assays for the lesser horseshoe bat were designed, targeting the ZFX, SRY and DBY genes. Using these sex typing assays and previously published microsatellite DNA markers, the sex ratio of adult bats was examined at six lesser horseshoe bat summer roosts across the species' range in Ireland using faecal DNA samples.

The lesser horseshoe bat's range in Ireland is limited to geographically isolated parts of the counties of the west coast. The population genetics of the species was examined using microsatellite genotyping of faecal DNA samples from 21 colonies from across its range, to assess the level of interbreeding and possible risk of inbreeding within this population.

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List of Abbreviations

A_R	Allelic richness
CMR	Capture-mark-recapture
Ct	Cycle threshold
Cyt b	Cytochrome b gene
DNA	Deoxyribonucleic acid
FAM	Fluorescein amidite
F_{IS}	Inbreeding coefficient
F_{ST}	Fixation index
H_2O	Water
H_E	Expected heterozygosity
H_O	Observed heterozygosity
HWE	Hardy-Weinberg equilibrium
JNCC	Joint Nature Conservation Committee
mtDNA	Mitochondrial DNA
µl	Microlitres
µM	Micromolar
N_A	Number of alleles
N_E	Effective number of alleles
NPWS	National Parks and Wildlife Service
PCR	Polymerase Chain Reaction
PI	Probability of identity
PI_{SIB}	Probability of identity of siblings
pmol	Picomoles
s	Seconds
Tm	Melting temperature
U	Undetermined

Chapter 1

General Introduction

1.1 Preface

This thesis consists of two main strands, one concerning the full set of bat species of the British Isles, and the other dealing more specifically with the lesser horseshoe bat, *Rhinolophus hipposideros*. Therefore, the scope of this introduction is quite broad, and although the specific aims of the thesis will be made clear at the end of the introduction, I felt it necessary to explain initially the need to cover this wide range of topics. This introduction is divided into three main sections: the first discusses the bat fauna of the British Isles, the second focuses on the lesser horseshoe bat, and the third comprises a discussion of the use of genetics in wildlife studies and how it can be applied to addressing the questions of interest in this study. The aims and objectives of the study will also be outlined.

1.2 Bats of the British Isles

1.2.1 Evolution and taxonomy

The bats (Chiroptera) form the second largest order of mammals after the rodents, comprising more than 1,300 described species worldwide (Fenton and Simmons, 2015). They have an almost global distribution, being absent only from the polar regions and isolated oceanic islands, and are most diverse in the tropics and subtropics. They inhabit a wide range of habitats in varying climates, including forests, wetlands, grassland and deserts, and they also exploit many food sources including fruit, nectar and pollen, invertebrates, vertebrates and (in three species only) blood (Kunz et al., 2011).

Due to the range of habitats and food sources that they exploit and their approximately 65 million year evolutionary history (Teeling et al., 2005; Tsagkogeorga et al., 2013), bats have highly divergent morphological adaptations and ecological niches. Despite this they are nevertheless united by several distinctive characteristics. They are the only mammals capable of true powered flight, on wings composed of skin stretched between the elongated fingers of the front limbs. They are exceptionally long-lived animals for their size, and reproduce very slowly, both adaptations partly being evolutionary consequences of the energetic constraints of flight, and the predator avoidance ability that it confers (Foley et al., 2018). Bats in temperate regions of the world tend to have

very similar life histories, spending a large part of the year hibernating in winter as they are unable to find enough food to sustain the energy cost of flight. The vast majority of bats are nocturnal and most species primarily depend upon echolocation, a form of sonar, to navigate and hunt at night (Dietz et al., 2009).

Bats were traditionally classified into two suborders: firstly, the megabats (Megachiroptera), which consist of the generally larger, fruit- or nectar-eating and non-echolocating species, such as flying foxes. The second recognised suborder was the microbats (Microchiroptera), consisting of the vast majority of bat species, which are generally smaller, nocturnal, insect-eating and echolocating (Teeling et al., 2005; Tsagkogeorga et al., 2013). However, molecular studies have suggested that this classification is incorrect, and have proposed a new classification scheme: the Yinpterochiroptera, containing megabats and several microbat families, including horseshoe bats; and Yangochiroptera, containing the remaining microbats, including the vesper bats which make up the majority of European bat species (Fig. 1.1) (Teeling et al., 2005; Tsagkogeorga et al., 2013). The bat species present on the British Isles are members of two families, the horseshoe bats (Rhinolophidae) and evening or vesper bats (Vespertilionidae) (Harris and Yalden, 2008).

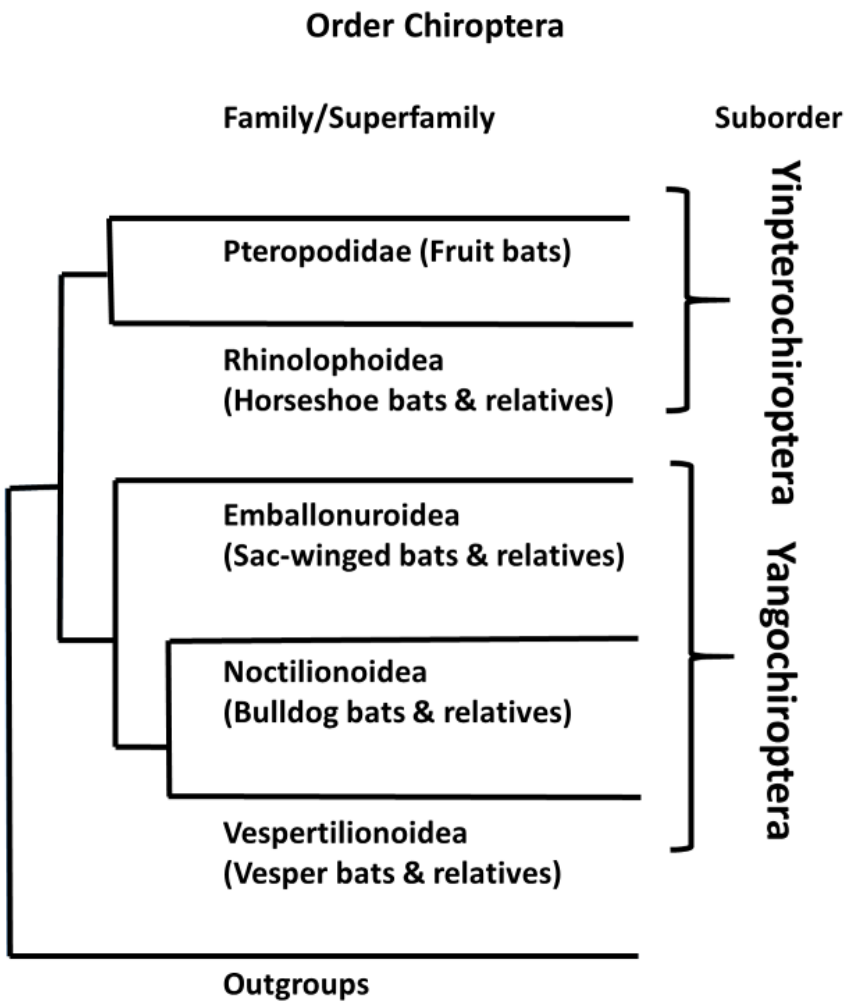

Figure 1.1: Modern phylogeny of bats (order Chiroptera), adapted from Teeling et al. (2005). Note that the single group formerly classified as "megabats", family Pteropodidae, is now classified in the same suborder as one of the groups once classified as part of the "microbats", superfamily Rhinolophoidea.

1.2.2 Ecological importance of bats

Bats are increasingly being recognised for their role in ecosystem functioning, including the maintenance and regeneration of tropical forests by fruit-eating bats which spread the seeds of trees in their droppings (Galindo-González et al., 2000; Kelm et al., 2008), and pollination of tropical flowers. Bats are responsible wholly or in part for the pollination of at least 528 plant species, including many economically valuable crops such as bananas, pineapples, avocadoes and agaves (from which tequila is produced), among others (Geiselman, 2010).

Bats are also extremely important worldwide due to their ecological role in controlling insect populations through the predation of insectivorous bat species. This is also of great benefit to agriculture through the removal of pest species; for example, Whitaker (1995) estimated that a colony of 150 big brown bats (*Eptesicus fuscus*) in the U.S. state of Indiana consumed approximately 1.3 million pest insects every year. It has been conservatively estimated that insect-eating bats in the continental USA are worth approximately $22.9 billion to agriculture every year through their effect on insect pests, although the true figure could be as high as $100 billion (Boyles et al., 2011). The impact of bats on insect pests in Europe appears to be comparatively less studied, but they are likely to be similarly important, including in the British Isles.

Bats can also be seen as important "bioindicator" species, as they are dependent on their insect prey populations, which are in turn currently vulnerable to the impact of habitat loss, pesticide use and climate change. Therefore, changes in bat populations can indicate wider negative (or positive) changes in the environment (Jones et al., 2009). For example, the presence of Daubenton's bat, *Myotis daubentonii*, on waterways in the UK has been shown to be useful as an indicator of water quality of rivers. In sufficient quantity, pollutants from sewage treatment works, industry and agriculture can severely reduce the diversity and quantity of aquatic insects. This reduction of the prey base of Daubenton's bats has been found to be linked to a reduced presence of this species on heavily polluted rivers (Langton et al., 2009).

1.2.3 Bat species of Great Britain and Ireland

At present, there are eighteen resident bat species in Great Britain and Ireland (Fig. 1.2, Table 1.1) (Mathews et al., 2018; Roche et al., 2014). Several other species have been recorded as vagrants in the British Isles, including the northern bat (*Eptesicus nilssoni*), Savi's pipistrelle (*Hypsugo savii*), Hoary bat (*Lasiurus cinereus*), pond bat (*Myotis dasycneme*), Geoffroy's bat (*Myotis emarginatus*), Kuhl's pipistrelle (*Pipistrellus kuhlii*), European free-tailed bat (*Tadarida teniotis*) and the parti-coloured bat (*Vespertilio murinus*) (Harris and Yalden, 2008; Bat Conservation Trust, 2010). However, these have not been included in this study and will not be considered further.

The bats of the British Isles consist of only two species of the Rhinolophid family (the greater and lesser horseshoe bats, *Rhinolophus ferrumequinum* and *R. hipposideros*),

with all of the remaining sixteen species belonging to the Vespertilionid family (Harris and Yalden, 2008). The Irish bat fauna forms a subset of that of Great Britain, with all eleven species recorded in Ireland known to be resident breeding species in Great Britain. Of these eleven species, nine are resident breeding species in Ireland: *Myotis daubentonii, M. mystacinus, M. nattereri, Nyctalus leisleri, Pipistrellus nathusii, P. pipistrellus, P. pygmaeus, Plecotus auritus and R. hipposideros.* The two remaining species, *M. brandtii* and *R. ferrumequinum*, have only been recorded as single individuals, most likely vagrants from Great Britain or continental Europe (Roche et al., 2014).

All bat species present in the British Isles are exclusively insectivorous, hunting their prey at night using echolocation. Their habitat preferences and hunting styles vary, but all will use trees, buildings, caves, disused mines and other underground sites as roosts in which to rest during the day and raise their young in summer, and for hibernation during the winter, as well as for "transitional" and mating roosts in spring and autumn (Roche et al., 2014).

As part of conservation efforts for the bat species of Ireland and Great Britain, an estimate of the population of each species is needed to assess the conservation status of each species and to provide a baseline for future monitoring of population trends. The most up-to-date population estimates for bat species in Ireland and Great Britain are included in Table 1.1. Population estimates for bats are based on a mixture of field surveys carried out as part of monitoring schemes and statistical techniques based on assumptions about the species in question (Mathews et al., 2018; NPWS, 2013).

For some species, the current population estimates are based on robust monitoring data and statistical analysis and therefore these are thought to be relatively reliable, e.g. for the greater horseshoe bat in Great Britain and lesser horseshoe bat in Ireland. For several species, such as the common pipistrelle in both Ireland and Great Britain, while good survey data from established monitoring schemes exists, population estimates are made by extrapolating from this data and are thus less reliable. Finally, for some species (e.g. whiskered bat, barbastelle bat) very little population information is available and population estimates are based on expert opinion; population estimates for these species are relatively unreliable and should be treated with caution, or a population estimate simply cannot be given (Mathews et al., 2018; NPWS, 2013). Therefore, the population

estimates given in Table 1.1 vary widely in their reliability and may change in the future.

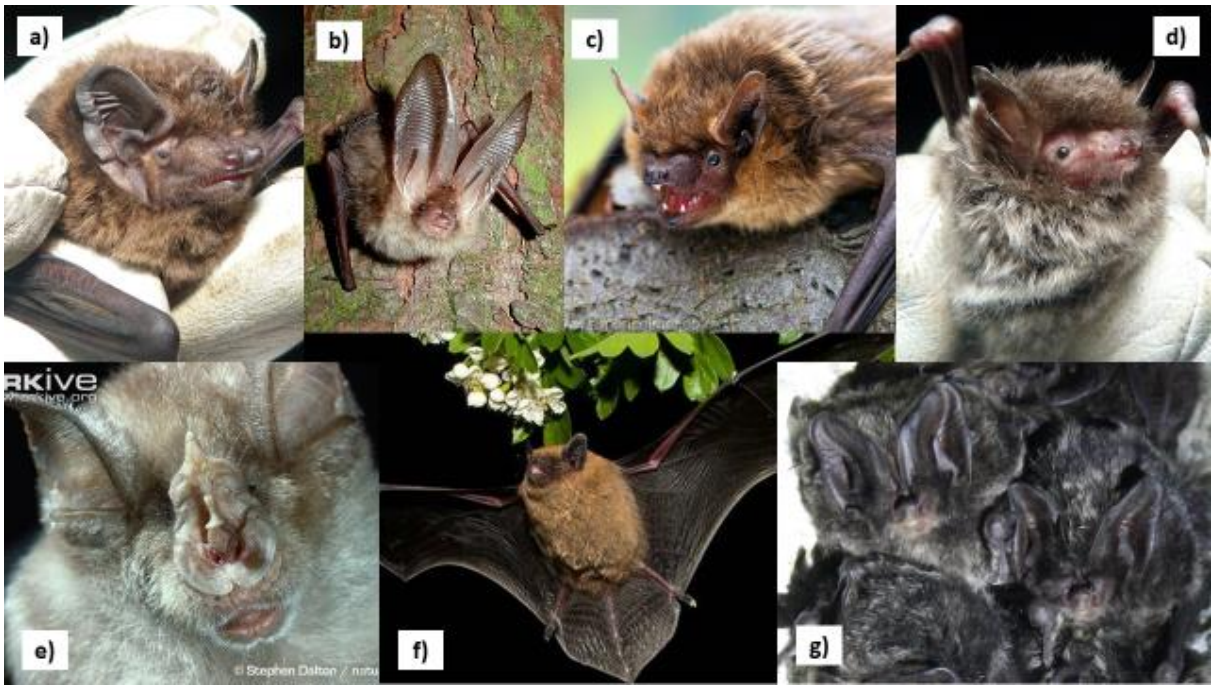

Figure 1.2: Representative species of the seven bat genera present in the British Isles: a) Leisler's bat, *Nyctalus leisleri* (Photo: Sam Dyer), b) brown long-eared bat, *Plecotus auritus* (John Altringham/Arkive), c) serotine bat, *Eptesicus serotinus* (Simon Colmer), d) Daubenton's bat, *Myotis daubentonii* (Sam Dyer), e) greater horseshoe bat, *Rhinolophus ferrumequinum* (Stephen Dalton/Arkive), f) common pipistrelle, *Pipistrellus pipistrellus* (Maurice Flynn), g) barbastelle bat, *Barbastella barbastellus* (Andrew Harrington).

Table 1.1: List of bat species resident in Ireland and Great Britain.

Species	Distribution-Ireland	Distribution-Great Britain	Foraging habitat	Roost sites	Population	IUCN Red list status
Barbastelle *Barbastella barbastellus*	Absent[1, 2]	Central & S England, Wales[2]	Old growth forest, rivers[2, 4]	Trees, disused buildings, caves, tunnels[2, 4]	5,000 (GB)[5]	NT[6]; VU (Eur)[6]
Serotine *Eptesicus serotinus*	Absent[1, 2]	Central & S England, Wales[2]	Pasture, woodland edge, hedgerow, parkland[2, 4]	Old buildings, trees, cliff crevices[2, 4]	136,000 (GB) [5]	LC[6]
Alcathoe *Myotis alcathoe*	Absent[1, 2]	Yorkshire, Sussex[3]	Riparian forest, especially in forested valleys[4]	Trees, caves[4]	2,000 (GB) [5]	DD[6]
Bechstein's *Myotis bechsteinii*	Absent[1, 2]	S England, SE Wales[2]	Old growth forest[2, 4]	Trees, caves, tunnels[2, 4]	21,800 (GB) [5]	NT[6]; VU (Eur)[6]
Brandt's *Myotis brandtii*	Vagrant? (Only 1 record) [1]	Widespread in England & Wales only[2]	Woodland, hedgerows, rivers[2, 4]	Buildings, trees, bridges, caves, tunnels[2, 4]	Unknown (Irl)[1], 29,500 (GB) [5]	LC[6]; DD (Irl)[1]
Daubenton's *Myotis daubentonii*	Widespread[1]	Widespread[2]	Rivers, lakes, rarely woodland[1, 2, 4]	Disused buildings, bridges, trees, caves, tunnels[1, 2, 4]	81,000 (Irl)[1], 1.03m (GB) [5]	LC[6]
Greater mouse-eared *Myotis myotis*	Absent[1, 2]	Formerly resident in S England[2]	Woodland, pasture[2, 4]	Buildings, caves, mines, tunnels[2, 4]	1 (GB) [5]	LC[6]
Whiskered *Myotis mystacinus*	Widespread[1]	Widespread except N Scotland[2]	Woodland, hedgerows, pasture, rivers[1, 2, 4]	Buildings, trees, caves, tunnels[1, 2, 4]	Unknown (Irl)[1], 40,000 (GB) [5]	LC[6]
Natterer's *Myotis nattereri*	Widespread[1]	Widespread except N Scotland[2]	Woodland, hedgerows, parkland[1, 2, 4]	Old buildings, trees, bridges, caves, tunnels[1, 2, 4]	Unknown (Irl)[1], 414,000 (GB) [5]	LC[6]

Leisler's *Nyctalus leisleri*	Widespread[1]	Widespread except N Scotland[2]	Pasture, woodland edge, water bodies[1, 2, 4]	Buildings, trees[1, 2, 4]	73,000 (Irl)[1], 24,000 (GB) [5]	LC[6]; NT (Irl)[1]
Common noctule *Nyctalus noctula*	Absent[1, 2]	Widespread to S Scotland[2]	Pasture, woodland edge, water bodies[2, 4]	Buildings, trees, cliff crevices[2, 4]	656,900 (GB) [5]	LC[6]
Nathusius' pipistrelle *Pipistrellus nathusii*	Widespread, possibly expanding[1]	Widespread except N Scotland[2]	Woodland edge, rivers, lakes[1, 2, 4]	Buildings, trees[1, 2, 4]	10,000 (Irl)[1], Unknown (GB) [5]	LC[6]
Common pipistrelle *Pipistrellus pipistrellus*	Widespread[1]	Widespread[2]	Woodland edge, hedgerows, pasture[1, 2, 4]	Buildings, bridges, trees[1, 2, 4]	1.2m (Irl)[1], 3.04m (GB) [5]	LC[6]
Soprano pipistrelle *Pipistrellus pygmaeus*	Widespread[1]	Widespread[2]	Woodland edge, hedgerows, rivers, lakes[1, 2, 4]	Buildings, bridges, trees[1, 2, 4]	540,000 (Irl)[1], 4.67m (GB) [5]	LC[6]
Brown long-eared *Plecotus auritus*	Widespread[1]	Widespread[2]	Woodland, scrub, hedgerows[1, 2, 4]	Buildings, trees, caves, tunnels[1, 2, 4]	64,000 (Irl)[1], 934,000 (GB) [5]	LC[6]
Grey long-eared *Plecotus austriacus*	Absent[1, 2]	S England[2]	Woodland, pasture, parkland[2, 4]	Buildings, caves[2, 4]	1,000 (GB) [5]	LC[6]
Greater horseshoe *Rhinolophus ferrumequinum*	Vagrant? (Only 1 record) [1]	SW England, Wales[2]	Woodland, hedgerows, pasture[2, 4]	Disused buildings, caves, tunnels[2, 4]	Unknown (Irl)[1] 12,900 (GB)[5]	LC[6]; NT (Eur)[1]
Lesser horseshoe *Rhinolophus hipposideros*	West coast, Counties Cork to Mayo[1]	SW England, Wales[2]	Woodland, scrub, hedgerows[1, 2, 4]	Disused buildings, caves, tunnels[1, 2, 4]	14,000 (Irl)[1], 50,400 (GB)[5]	LC[6]; NT (Eur)[1]

Table legend: IUCN Red List Status: DD- Data Deficient; LC- Least Concern; NT- Near Threatened; VU- Vulnerable.

References: 1) Roche et al., 2014; 2) Harris and Yalden, 2008; 3) Jan et al., 2010; 4) Dietz et al., 2009; 5) Mathews et al., 2018; 6) IUCN, 2015.

Notes: Where a range of population size estimates is given in the literature, the lower estimate is shown here. Red list status shown is for global assessment, assessments for Ireland, UK or Europe included only when they differ from this. Compass points abbreviated for geographical locations (e.g. SW England- south-western England).

1.2.4 Threats from human activities

As with many other species, bats are vulnerable to and are becoming increasingly affected by various human activities in the landscape. These can be broadly broken down into direct mortality of bats, such as that caused by wind turbines; changes in suitability of habitats for bats, for example caused by habitat fragmentation or destruction, and disturbance of bats by artificial lighting; and interference with or destruction of bat roost sites (Jones et al., 2009; Mathews et al., 2018; Roche et al., 2014).

One of the most serious threats to bat populations is the disturbance or destruction of their roosts. Bats are known to use several different types of structures to roost in, including natural features such as crevices and hollows in trees, caves, and crevices in rock faces and cliffs. Many man-made structures are also used by bats (in many species now forming the main source of roost sites), such as houses and other buildings, bridges, cellars, tunnels and mines. The type of roost used tends to vary according to the species of bat and the time of year; bats use different roost sites in which to hibernate, to rear their young, for mating and other purposes according to the different roost temperatures they require (Roche et al., 2014).

Due to their often close proximity to humans, bat roosts are highly vulnerable to disturbance. Disturbance can be caused by the lighting of roost entrances, by people entering the roost at inappropriate times or by inappropriate building work on roost sites. In particular, female bats are very sensitive to disturbance of their roosts during the breeding season (usually June-mid August) and in serious cases this can cause them to abandon their young. Bat roosts can be completely destroyed by human activity due to the demolition or extensive renovation of roost sites, or by the deliberate or inadvertent blocking of roost exits. Such cases can often entomb and kill an entire bat colony, or cause the loss of the roost site and force the bats to move to another, often less suitable, location (Roche et al., 2014). Due to their vulnerability, bat roosts have been given special legal protection under wildlife legislation in both the Republic of Ireland and the United Kingdom (NPWS, 2009; Schofield, 2008).

1.2.5 Legal protection of bats

As the threats to bats from the intensification of agriculture (particularly the use of pesticides) and the loss of roosts became increasingly recognised in previous decades, bats have been accorded a high level of legal protection at a national and international level. All bat species and their resting places (i.e. roosts) are protected in Irish law under the Wildlife Act, 1976

(amended 2000) (NPWS, 2009; Roche et al., 2014). It is an offence to disturb or destroy bat roosts whether or not bats are present at the time, to disturb bats at their roost or to capture bats without a licence from the National Parks and Wildlife Service (NPWS). This act also allows for the creation of Natural Heritage Areas (NHAs) to protect sites of local and national biodiversity interest, and several dozen sites have been proposed for NHA status due to the presence of important bat roost sites. However, the majority of sites selected for NHA status in Ireland have not yet been fully ratified as such and remain as proposed NHAs (pNHAs); these sites have little protection under the Wildlife Act, although they have some limited protection under planning acts and local authority regulations (NPWS, 2018), so they remain at risk of disturbance, damage or destruction.

In the UK, bats are protected under the Wildlife and Countryside Act, 1981, which makes it an offence to disturb or destroy bat roosts or to obstruct bats' access to a roost, and provides additional protection to Special Sites of Scientific Interest (SSSIs), which had been set up under earlier legislation, and many of which have been designated for their bat populations (Harris and Yalden, 2008, Schofield, 2008). Both the Irish and UK legislation requires special licences to be held by people wishing to carry out several activities relating to the study of bats, including disturbance of roosts, capturing live bats, collection of tissue samples from live bats and the possession of dead bats, among others (Harris and Yalden, 2008).

At the international level, bats are protected at a high level by EU law under the Habitats Directive of 1992. This directive is aimed at protecting habitats and species (except birds) which are of conservation interest or are under threat within the EU. Within this directive are several categories offering different levels of protection to particular species. Annex IV of the habitats directive lists all species which are to be strictly protected within the European Union; this section includes all bat species present in Europe (Roche et al., 2014). In addition, Annex II lists particular species for which Special Areas of Conservation (SACs) must be designated as protected areas within member states of the EU where such species exist in important numbers for the EU as a whole, and so as to protect areas of suitable habitats which these species depend on. Fourteen bat species are included in Annex II, out of a total of 51 bat species currently recorded in the western Palaearctic as a whole (Dietz et al., 2009). Five of these species occur in the British Isles: the greater and lesser horseshoe bats, *R. ferrumequinum* and *R. hipposideros*; the barbastelle bat, *Barbastella barbastellus*; and Bechstein's bat and the greater mouse-eared bat, *M. bechsteinii* and *M. myotis*. In Ireland, 41 SACs include lesser horseshoe bats as a "qualifying interest", including small sites designated specifically for their

use as a roost by the bats (e.g. old buildings, ice houses, caves) and larger sites where the bat is just one of a range of species of interest (e.g. Killarney National Park) (NPWS, 2018). None of the SACs present in Northern Ireland include bats as a qualifying interest, as none of the Annex II bat species are known to occur there (JNCC, 2015). In Great Britain, SACs have been designated for the lesser horseshoe bat (15 SACs), greater horseshoe bat (11), barbastelle bat (9) and Bechstein's bat (9) (JNCC, 2015). Additionally, EU member states are required to report on the current conservation status and population trends of Annex II species (including all bat species) to the European Commission once every six years under Article 17 of the Habitats Directive (JNCC, 2013; NPWS, 2013).

1.2.6 Surveying for bats

According to the wildlife legislation which protects bat species in Ireland and the UK, bats and their roosts are strictly protected in both countries, as has been mentioned in Section 1.2.5. Therefore, activities which could adversely affect or destroy bat roosts are prohibited or strictly controlled, requiring a derogation licence to be granted by the appropriate wildlife authority (e.g. the NPWS in Ireland) (Aughney et al., 2008). Planning permission for building activities on structures which could potentially contain a bat roost, and derogation licences where a bat roost has been found, are usually only granted subject to an extensive survey of the structure in question by a bat worker to minimise the impact of such works. Such a survey is necessary to identify whether a roost is present, the potential for disturbance to be caused to the roost by the proposed works, and mitigation measures to prevent disturbance to the bat colony while allowing works to go ahead, where this is possible (Aughney et al., 2008).

The aims of a bat survey of a structure are to establish if the structure is indeed a bat roost, which species occupies the roost, what type of roost it is (e.g. maternity, hibernation) and how many bats are present (Aughney et al., 2008). All of these are important questions when it comes to determining the relative importance to local bat populations of a particular roost site, and the mitigation measures which can be implemented to prevent disturbance to the roost in question. In order to answer these questions, it is usually necessary to apply several different survey methods to gain a complete understanding of the activity of bats at a particular roost (Aughney et al., 2008).

A survey of a potential bat roost usually begins with a daytime survey of the site, involving a thorough inspection of the entire structure to search for bats or their field signs. Site inspections

can indicate the presence of bats by identifying their field signs, including scratch marks and grease stains at roost exits, prey remains (e.g. moth wings) and bat droppings inside the roost itself or outside exits. Bats themselves can sometimes be found inside roosts, but this is dependent on the species as some tend to hide deep in cracks in masonry or woodwork. The appearance of bat droppings may also be indicative of the species present, but the droppings of some groups of species (e.g. pipistrelles) may be so similar that it is impossible to tell with certainty which is the species of origin. In mixed species roosts there is also scope for confusion, or the obscuring of the presence of small numbers of one species by the overwhelming number of droppings of another species present in greater numbers at the same roost (Aughney et al., 2008).

In the case of most species (except perhaps horseshoe bats, *Rhinolophus* spp., which hang freely from ceilings and thus are easier to count within roosts), an interior inspection of a building is usually combined with night-time surveys to provide further information on bat roosts where they are discovered (Aughney et al., 2008). This involves emergence surveys where bats are counted exiting the roost and their calls are recorded for later analysis, which can be used to identify the bat species present. However, although bat sound recording gear is becoming ever more sophisticated, as with identifying bat droppings by eye identifying species by their calls can be difficult for some groups, for example the *Myotis* species. In certain cases some cryptic bat species such as whiskered/Brandt's are very hard to distinguish by sound analysis (Ahlén and Baagøe, 1999).

Cryptic bat species are two (or more) bat species which are very similar or almost identical to each other in their morphology, echolocation calls and ecology but are genetically distinct. One of the first sets of cryptic species definitively distinguished from each other with the help of genetic analysis were the common and soprano pipistrelles, *Pipistrellus pipistrellus* and *P. pygmaeus* (Barratt et al., 1997; Racey et al., 2007). Cryptic species pairs are often not especially closely related, as in the case of the whiskered bat (*Myotis mystacinus*) and Brandt's bat (*Myotis brandtii*), which although they appear almost identical physically are in fact only distant relatives, belonging to different subgenera within *Myotis* (Ruedi and Mayer, 2001).

In cases where cryptic bat species are suspected to be present, it may be necessary to trap bats, either capturing them within the roost space if this is accessible, or by netting them while exiting the roost. While most species can be identified in the hand by experienced bat workers, this may not be enough to identify some cryptic species as they can be almost identical in the hand and a DNA test may be the most reliable way of distinguishing which species is present

(Boston et al., 2010). The application of DNA technology to the identification of bat species will be discussed in more detail in Section 1.4. DNA identification and monitoring of bat species also has the advantage of the potential to reduce the need for more invasive survey methods such as entering roost spaces and especially trapping of bats, both of which require licensing from the appropriate wildlife authority (Boston et al., 2012).

Bat droppings are a readily available source of DNA which can be collected non-invasively (i.e. without direct disturbance to bats) and have been used in some genetic studies of European bats (Boston et al., 2012; Puechmaille et al., 2007; Puechmaille and Teeling, 2014). However, non-invasive samples have been used in relatively few genetic studies of European bat species and there appears to have been little emphasis in the literature on developing DNA testing methods for the identification of European bat species. A complete set of DNA tests to identify the bat species of the British Isles would be a useful additional tool for bat surveyors and researchers. Therefore, one of the two main strands of this thesis is concerned with designing and testing a set of DNA primers for the British bat fauna.

1.3 The lesser horseshoe bat

1.3.1 Introduction

The lesser horseshoe bat, *Rhinolophus hipposideros*, (Fig. 1.3) is a member of the Rhinolophidae, or the horseshoe bat family, so-called because of the horseshoe-shaped flaps of skin, or nose-leaf, surrounding their nostrils. It is a small bat species, weighing only 5-7 grams, being one of the smallest species in Europe (Dietz et al., 2009).

Figure 1.3: A lesser horseshoe bat, *Rhinolophus hipposideros* (Photo: Helder Conçeiçao).

The lesser horseshoe bat occurs across most of Europe, from Ireland and Iberia in the west, east to the Black Sea and the Caucasus, with a northern limit in a line running roughly from the west of Ireland, across northern Germany to southern Ukraine. It is also present in parts of North Africa and western and central Asia as far east as Kashmir in the western Himalayas (Fig. 1.4) (Dool et al., 2013; Taylor, 2016a).

As it lives across a large geographic range, the habitats it uses vary. However, in Europe it is mainly associated with wooded habitats including broadleaf forest, scrub, orchards and wooded river corridors, and it actively avoids open areas, travelling instead along hedgerows and treelines (Bontadina et al., 2002; Dietz et al., 2009). It is adapted to hunting within dense vegetation and "clutter", with short, broad wings which give it the agility to travel and forage effectively within this habitat, often flying directly through the canopy of trees (Bontadina et al., 2002).

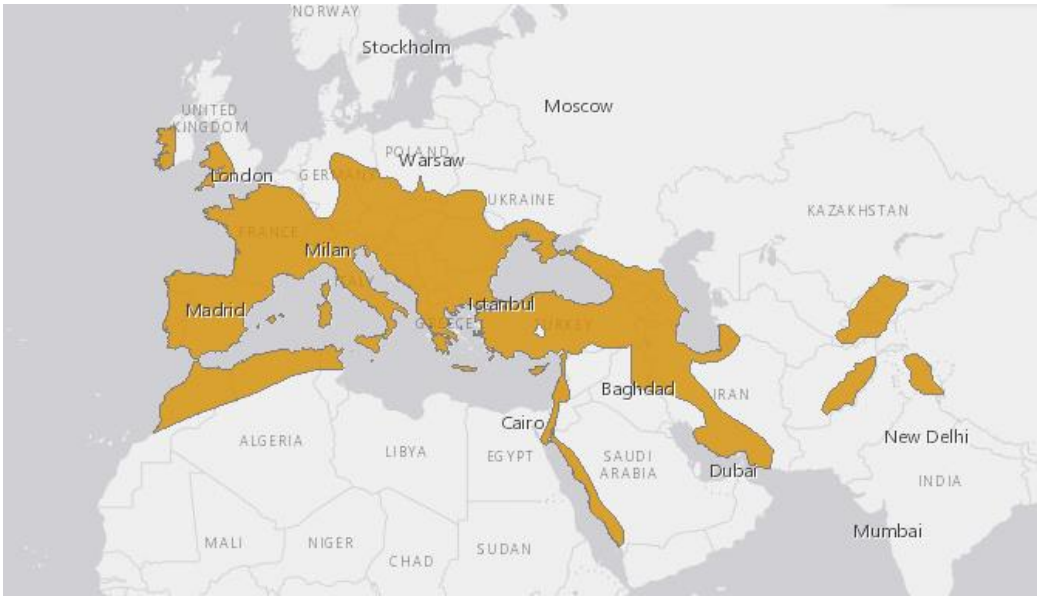

Figure 1.4: World distribution of the lesser horseshoe bat, *Rhinolophus hipposideros* (IUCN red list map).

Like other "microbat" species, the lesser horseshoe bat uses echolocation for navigation and to find its prey, producing ultrasonic calls (high pitched sounds above 20 kHz, beyond the range of human hearing) which bounce off its surroundings and allow it to find its way from the pattern of echoes produced, analogous to sonar used by submarines. However, the two British *Rhinolophus* species (as with all species in this genus) produce echolocation calls which differ in several respects from other British bats. Rhinolophids echolocate through the nose, with horseshoe-shaped flap of skin and other protrusions around the nose helping to direct the echolocation call into a narrow "beam" of sound, making their calls highly directional. Secondly, they depend on a property of echoes called "Doppler shift", where the pitch of a call echo returning from a prey insect changes in pitch from the outgoing call depending on whether prey is moving towards or away from the bat. Rhinolophids also echolocate at a much higher frequency than other British bats. The lesser horseshoe bat has the highest frequency echolocation calls of any bat species in the British Isles, at approximately 110 kHz. This allows it to obtain greater information from its surroundings than lower frequency calls, which provide poorer resolution (Dietz et al., 2009; Long and Schnitzler, 1975; Schofield, 2008).

Its agile flight and echolocation characteristics (highly directional and high resolution, can sense direction of movement of prey) allow the lesser horseshoe bat to hunt effectively in the dense vegetation of wooded habitats. Its prey consists of a broad variety of small insects and other invertebrates, including midges, mosquitoes, craneflies and other dipteran flies, moths,

beetles, caddisflies and spiders. These are either caught and consumed in flight, or sometimes gleaned directly from vegetation (Harris and Yalden, 2008; McAney et al., 2013).

Due to a poorly developed pelvis, the lesser horseshoe bat has difficulty crawling, meaning that it cannot pass through narrow gaps in search of roosting sites (McAney et al., 2013). Therefore, it needs roosts that it can fly directly into, where it hangs freely from the roof like other horseshoe bats. Its favoured roosting sites in Europe include caves (probably originally its main source of roosts) and man-made tunnels, a variety of building types such as buildings with attic spaces or cellars, or stables and other outbuildings, and more rarely large rot holes in old trees (Harris and Yalden, 2008; McAney et al., 2013). In summer, female lesser horseshoe bats gather at maternity roosts where they give birth to a single pup, usually between mid-June and mid-July, with the young bats becoming independent within six weeks of birth (Reiter, 2004; Schofield, 2008).

1.3.2 Evolutionary history

The lesser horseshoe bat is one of at least 77 known species in the genus *Rhinolophus*, distributed across Eurasia, Africa and Australasia occupying ecosystems in tropical, subtropical and temperate climates (Dietz et al., 2009; Stoffberg et al., 2010). This genus is believed to have appeared in Asia approximately 50 million years ago, during the Eocene epoch, and is the only genus within the family Rhinolophidae (Stoffberg et al., 2010).The Rhinolophids are most closely related to the leaf-nosed bats (Hipposideridae), and both families form part of the suborder Yinpterochiroptera, which also includes the flying foxes or fruit bats (Teeling et al., 2005; Tsagkogeorga et al., 2013).

Of the 77 species of *Rhinolophus* bats, only five occur in Europe. The Mediterranean horseshoe bat (*R. euryale*), Mehely's horseshoe bat (*R. mehelyi*) and Blasius' horseshoe bat (*R. blasii*) are all largely restricted to the Mediterranean basin in the European part of their respective ranges. However, the greater horseshoe bat (*R. ferrumequinum*) and the lesser horseshoe bat are more widely distributed in Europe, extending into much of central and northern Europe. The lesser horseshoe bat is the most widely distributed of the five species in Europe (Taylor, 2016a).

The lesser horseshoe bat is thought to have evolved in western and central Asia, from where it colonised south-eastern Europe sometime during the Pliocene epoch (5- 2.5 million years ago), which is supported by both fossil evidence and molecular phylogeographic analysis (Dool et al., 2013). The cycle of successive glacial periods during the Pleistocene epoch (2.5 million to

11,700 years ago) caused the lesser horseshoe bat to periodically retreat to temperate climate "refugia" in southern Europe, followed by recolonisation of central and northern Europe during warmer interglacial periods. Long periods of isolation in refugia in Iberia, southern Italy and the Balkan Peninsula have resulted in genetically distinct populations in these areas (Dool et al., 2013).

Lesser horseshoe bats recolonising central and northern Europe since the Last Glacial Maximum (LGM), which occurred approximately 20,000 to 18,000 years ago, appear to have originated largely in the Balkan refugium. Bats present in refugia in Iberia and southern Italy appear not to have expanded from these areas after the LGM, possibly due to the presence of physical barriers such as the Pyrenees. Thus, the ultimate origin of the current lesser horseshoe bat populations in Ireland and Great Britain lies in the Balkan Peninsula, via central Europe (Dool et al., 2013).

1.3.3 The lesser horseshoe bat in Ireland

The lesser horseshoe bat is one of Ireland's rarest bat species. It is the only member of the Rhinolophid family (i.e. horseshoe bats) resident in Ireland, in contrast to the other eight resident Irish bat species which all belong to the Vespertilionid family (or vesper bats).

The past history of the lesser horseshoe bat in Ireland is largely unknown. As a woodland specialist species, it probably could not have colonised Ireland at least until the first scrubby woodlands of hazel, birch and juniper had regenerated across the island after the last glacial period, by about 9,000 years ago (Mitchell, 2006). The only known lesser horseshoe bat fossil remains in Ireland were discovered in a limestone cave at Kilgreany, near Dungarvan in County Waterford (Movius et al., 1935). This cave contains remains of a wide range of wild animals, humans and domestic animals, deposited over a very long period of time. These lesser horseshoe bat remains have never been radiocarbon dated but were found amongst reindeer (*Rangifer tarandus*) bones radio carbon dated to about 10,700 years ago, and two sets of human remains dated to approximately 4,800 and 4,500 years ago, respectively (Molleson, 1986). Thus, the lesser horseshoe bat appears to be a long-established species in Ireland, most likely having colonised via the east coast of Ireland from the nearest population in Great Britain (Dool et al., 2016).

The single fossil record from County Waterford lies far to the east of its present range in the counties along the west coast, indicating that it was once more widely distributed across the

island. Suitable forest habitat would once have been present over almost the entire island, but the gradual deforestation of the country for the purposes of farming since the Neolithic period (from about 4200 BC onwards) has probably been the primary cause for the contraction of the lesser horseshoe bat's range (Dool et al., 2016). By the mid-1600s overall forest cover in Ireland had been reduced to somewhere between 3% and 12.5%, comparable to today's figure of about 11.5% woodland cover (Eurostat, 2013; Hickey, 2011). However, some areas were still densely forested with native broadleaf trees, especially parts of the west and the large river valleys of the south of Ireland (Hickey, 2011), possibly providing sufficient natural habitat for a more extensive lesser horseshoe bat range than today.

From the mid-1600s, Ireland's remaining woodlands were almost completely eradicated. This was largely due to timber extraction for industrial activity, especially for barrel making and charcoal burning to fuel iron smelting, and woodland clearance by a booming human population who required farmland and fuel. By about 1918 much of Ireland was almost completely denuded of all its woodland, scrub and hedgerows, with less than 0.5% of the country covered with woodland of any kind (including non-native conifer plantations). After this period, woodland and scrub cover began to gradually increase again (Mitchell and Ryan, 2007).

Although there is no direct evidence, it is likely that this almost complete loss of its favoured habitat contributed to the dwindling of the lesser horseshoe bat's range to several small clusters in the west of Ireland. Of the few woodlands that remained in the country, most were located within the estates of the landed gentry, who preserved pockets of ancient woodland (defined in Ireland as woodland in continuous existence since at least 1650) and planted new woodlands (Mitchell and Ryan, 2007). Many lesser horseshoe bat roosts are still located within or near former or still-existent large estates with plentiful woodland cover. Examples include Glengarriff Demesne in County Cork, now Glengarriff Nature Reserve; the Kenmare and Muckross Demesnes in County Kerry, which now make up the core of Killarney National Park; Curragh Chase Demesne in County Limerick, now managed by Coillte (the Irish state forestry company) for recreation and wildlife; and Coole Demesne in County Galway, now Coole-Garryland Nature Reserve.

When the first scientific reports of the occurrence of the lesser horseshoe bat in Ireland were made from 1858 onwards, it was already clear that this species only occurred in the west by that time, and well into the 20th Century it had only been recorded in four counties- Cork, Kerry, Clare and Galway (Moffat, 1938). Therefore, the major contraction of this species' range to

the west of Ireland had probably already occurred prior to the 1800s. Its distribution and population size was still poorly understood until more thorough surveys began to be undertaken from the 1970s onwards (Kelleher, 2004).

The lesser horseshoe bat is now known to be resident only in parts of six counties along the west coast- Cork, Kerry, Limerick, Clare, Galway and Mayo (Fig. 1.5). Even within the six core counties it is limited to certain localities, and does not form a single continuous range, instead forming four main geographically separated subpopulations: one present in west Cork and south Kerry, mainly in the Iveragh and Beara Peninsulas; a small population in central Limerick; one in central Clare and south Galway; and another population present in north Galway and south Mayo, with some scattered roosts near Galway City connecting it to the south Galway-Clare population. The lesser horseshoe bat has the smallest range of the resident bat species in Ireland (Roche et al., 2014). Small numbers of lesser horseshoe bats or their droppings have been found in recent years in Counties Roscommon and Sligo, and mid-County Cork, places where the species has not been found before, which indicates that this species may be starting to expand its range into these areas (McAney, 2014).

The lesser horseshoe bat's favoured habitat in Ireland broadly consists of hilly countryside with extensive areas of broadleaf woodland or scrub which are well-connected by hedgerows. However, the landscapes occupied by this species in Ireland vary from deep mountain valleys and extensive broadleaf forests in Kerry and West Cork, limestone karst terrain with hazel scrub in Clare and South Galway, and intensive lowland farmland with small patches of woodland in Limerick. As this is a forest-adapted bat species, it depends on the presence of such wooded areas within the landscape to provide foraging areas.

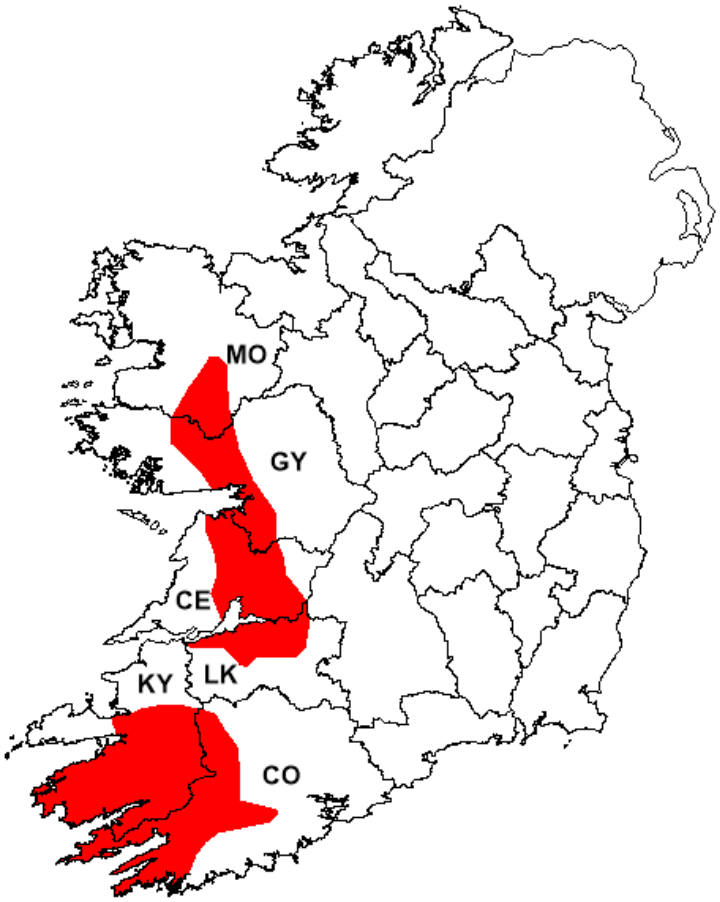

Figure 1.5: Map showing the approximate distribution of the lesser horseshoe bat in Ireland, marked in red. Some scattered records are known from elsewhere, but the main breeding population is located within this area. Large gaps between known breeding sites may not be apparent in this map, as in the case of the geographically isolated population in County Limerick. Counties within the lesser horseshoe bat's main range are labelled (CO-Cork, KY-Kerry, LK-Limerick, CE-Clare, GY-Galway, MO-Mayo).

The lesser horseshoe bat in Ireland was previously sometimes described as "the bat of the aristocracy" due to its propensity to roost in the large country houses of the landed gentry (Kelleher, 2006). However, since the early 20th Century, many such houses have been destroyed or abandoned and fallen into ruin, and the horseshoe bats have been forced to roost elsewhere. In summer, horseshoe bats require roosts for several different purposes, including maternity roosts where pups are reared, small satellite roosts near larger colonies, transitional roosts in use between the summer period of activity and winter hibernation, and night roosts used as resting places during nightly hunting forays. Important factors in the choice of roost are sufficient darkness, accessibility (bats must be able to fly straight in), and a stable, warm temperature and high humidity, but often sub-optimal sites which do not provide ideal conditions must be used (Kelleher, 2006). Main summer roosts can be found in castles,

mansions, cottages and houses of various kinds, farm buildings such as old stables, and churches and abbeys; there is also one record of a large summer roost in a hollow tree in County Kerry from the 1930s (Kelleher, 2006; Roche et al., 2014). The sites used are usually those constructed before 1900, built of stone and roofed with slate or sometimes corrugated iron, and in many cases are disused or rarely frequented by humans, where the bats are left undisturbed. In most sites, bats roost in the warmer attic or upper floors of buildings. Night roosts have been found in more variable places offering temporary shelter, such as upturned water tanks, tree hollows and derelict cottages (Kelleher, 2006; Roche et al., 2014).

During the winter hibernation period, lesser horseshoe bats usually roost in underground structures, which provide cool, stable temperatures and high humidity levels needed by this species. Some buildings mainly used as summer roosts are also used for hibernation, where cool, dark and humid ground-floor rooms are available. Much of this species' range in Ireland coincides with areas of limestone bedrock, which tends to form extensive networks of caves which are often used by these bats for hibernation. It also uses manmade underground structures including cellars, ice houses, tunnels and abandoned mines (McAney et al., 2013; Roche et al., 2014).

1.3.4 Conservation issues and legal protection

Until the mid- 20th Century, the lesser horseshoe bat was considered to be a common bat species across much of Europe. However, from the 1950s, along with many other bat species, it began to undergo a massive population decline in many parts of Europe. Lesser horseshoe bat populations in western and central Europe were especially badly affected. It went extinct in the Low Countries and most of Germany and Poland, and populations are known to have declined dramatically in south-eastern Germany, Switzerland, Austria, France and the Czech Republic (Taylor et al., 2016a). This decline is thought to been caused mainly by the intensification of agriculture in these areas during the course of the 20th Century. This entailed the destruction of semi-natural habitats and the use of highly toxic pesticides such as DDT, thereby destroying the horseshoe bat's habitat and wiping out its insect prey. The use of pesticides (DDT, lindane) as wood treatments in roost buildings also had a major impact by directly poisoning bats, and urban sprawl may also have been a factor (Bontadina et al., 2000). In some areas that were affected such as Switzerland, lesser horseshoe bat populations have been slowly recovering since the early 2000s (Taylor, 2016a).

In Great Britain, the lesser horseshoe bat is now present only in south-western England and most of Wales. It was once more widespread, as it was known to be present in Yorkshire and Derbyshire in north-central England and along the south coast of England until the early 20th Century, but has since disappeared from these areas (Schofield, 1996).

As described in the previous section, Ireland's lesser horseshoe bat population probably declined at an earlier stage than on the continent, most likely due to extensive deforestation. Its range broadly appears not to have changed to a great extent since the mid-1800s, but nothing is known of its population trends prior to the 1970s. A nationwide survey of horseshoe bat roosts was carried out by the Vincent Wildlife Trust between 1999 and 2004 to identify previously unknown roosts and to revisit roosts previously discovered but which had not been visited for some time. The survey noted the decline or disappearance of bats from a number of roosts which had previously been surveyed in the early 1990s. However, it appeared that the deterioration of the old and often disused buildings being used as roosts was the primary cause of roost abandonment instead of agricultural intensification as happened on mainland Europe, and the bats were possibly moving to different roosts in the same area in at least some cases (McAney et al., 2013).

As a result of this decline across Europe, the lesser horseshoe bat has been given legal protection at several levels to help conserve its remaining population. The legal protection given to bats has been discussed in detail in Section 1.2.5. In summary, the lesser horseshoe bat and its roosts are protected by law in Ireland under the Wildlife Act 1976 (as amended). It is also protected under the EU Habitats Directive, and as a species listed in Annex II, part of its range in Ireland is protected by 41 Special Areas of Conservation (SACs). Every six years, the NPWS is required to prepare a population estimate and conservation assessment for the lesser horseshoe bat as part of Article 17 reports submitted to the EU under the Habitats Directive, most recently in 2013. The monitoring carried out to allow a population estimate for this species will be discussed in more detail in the following section.

The Irish lesser horseshoe bat population's conservation status has been assessed as "least concern" in Ireland's Red List of Terrestrial Mammal Species (Marnell et al., 2009). The most recent Article 17 report for Ireland has also assessed the horseshoe bat's overall conservation status as "favourable" (NPWS, 2013). Monitoring of this species since the 1980s has shown that its range has remained relatively stable, its current population of 14,000 has shown a gradual increase over that period, and a substantial part of its population (at least 5,000 animals) is protected by being located within SACs designated for lesser horseshoe bats. Within its

range, habitat suitable for this species (e.g. woodland, scrub, watercourses, hedgerows) makes up approximately 30% of the land area overall and has remained stable at this level. Overall, the report argued that the evidence for gradual population increase, general stability of range and habitat availability, and positive future prospects for the species justified the favourable assessment (NPWS, 2013).

However, the Article 17 report acknowledges that the lesser horseshoe bat continues to face threats in Ireland, albeit ones that it deems to be manageable, and other threats have been highlighted by other authors (NPWS, 2013). The unsympathetic management of woodlands, including clear-felling of forestry plantations, the destruction of hedgerows and other field boundaries and the clearing of scrub in farmland all reduce the quantity of foraging habitat and make commuting between roosts and hunting grounds more difficult for this species (NPWS, 2013). The construction of large motorways through parts of its range have also destroyed habitat and have created a barrier which horseshoe bats find difficult to cross (Abbott et al., 2012). Despite the ongoing risk of habitat destruction from these sources, woodland cover is generally increasing in Ireland (although most of this is non-native conifer forestry), potentially providing new habitat for this species, and new motorways generally provide underpasses and other mitigation measures to allow for continued habitat connectivity for bats and other wildlife (Abbott et al., 2012; NPWS, 2013).

The loss or damage of roosts is another major threat to this species in Ireland. While some damage to roosts has been caused through human activity, one of the main factors in the decline or abandonment of roosts is the deterioration of the disused buildings used most by the bats (NPWS, 2013; Roche et al., 2015). Roche et al. (2015) collated assessments of summer and winter lesser horseshoe bat roosts in Ireland, categorised as "good", "unfavourable-inadequate" and "unfavourable-bad". They found that 44 out of 92 (48%) summer sites assessed from 2013-2014 and 15 out of 76 (20%) winter sites assessed from 2014-2015 were in either inadequate or bad condition. Roost assessments included aspects such as loss of surrounding foraging habitat and commuting routes, and unexplained population declines, but the authors highlighted the importance of roost deterioration or damage, such as rain ingress into roofs and other effects of the ageing of buildings, natural disasters such as flooding of caves, or deliberate damage such as dumping of rubbish at cave entrances. They also noted that some roosts in good condition experienced large population increases while nearby roosts in inadequate or bad condition had population declines, suggesting migration of bats to roosts in better condition (Roche et al., 2015).

While the poor state and ongoing deterioration of many roosts poses a serious risk to this species, Roche et al. (2015) state that even a small amount of money can go a long way to maintaining and even improving buildings or other sites for use as lesser horseshoe bat roosts, such as roof repairs or boarding up of windows and insertion of partitions at entrances to make the roost interior darker. Some buildings located within national parks or nature reserves and thus owned by the state have been maintained or even renovated specifically for use by lesser horseshoe bats, including three summer roosts present in Killarney National Park (pers. obs.). A small number of new roosts have also been built in some protected areas, including purpose-built underground hibernation sites constructed in Glengarriff Nature Reserve and Coole-Garryland Nature Reserve, which have been readily occupied by lesser horseshoe bats in these areas (pers. obs.). In addition to the NPWS, the Vincent Wildlife Trust has also purchased (or leased) and extensively repaired 13 buildings containing lesser horseshoe bat roosts in Ireland. The extensive repair work has had a positive effect, with the population of many of these sites increasing steadily. The summer roost with the largest population in the country, with 420 bats counted in summer 2014, is a VWT reserve in Co. Kerry, and VWT reserves contained just over 3,900 bats in summer 2016, approximately 27% of the estimated national population of 14,000 (Roche et al., 2015; VWT, 2017).

Most recently, two genetic studies of the lesser horseshoe bat population in Ireland have highlighted the threat posed by inbreeding in isolated subpopulations, which puts affected populations at greater risk of extinction. The Irish lesser horseshoe bat population is completely isolated from the nearest neighbouring populations in Great Britain and north-western France, and has the lowest level of genetic diversity across the entire species' range (Dool et al., 2013). Additionally, the Irish lesser horseshoe bat population is split into two distinct, isolated subpopulations, one in Cork and Kerry and the other made up of all other horseshoe bats to the north, from Limerick to Mayo (Dool et al., 2016). The southern subpopulation in Cork and Kerry is larger numerically, but the northern subpopulation is more diverse genetically (Dool et al., 2016). The two populations are separated by a gap of approximately 25 km between the nearest recorded roosts in Counties Kerry and Limerick, separated only by a low mountain range on the Kerry-Limerick border. However, many horseshoe bat roosts discovered in County Limerick in the 1980s now appear to be unoccupied, and the remaining occupied roosts centred on the Curraghchase Estate are about 50 km from the nearest Kerry roost (Roche, 2001). There is no impassable physical barrier between the two populations, and there is some evidence that the dereliction or demolition of suitable roosting sites and destruction of

woodland habitat has played at least some part in the decline of horseshoe bats in this area and the opening of a gap across which interbreeding cannot occur (Roche, 2001; Dool et al., 2016). While the Limerick horseshoe bats appear to be part of the broad "north-range" subpopulation, they are located approximately 30 km overland from the nearest population in south-east Co. Clare, with Limerick City and the estuary of the River Shannon forming barriers in between. Both winter and summer roosts in south-eastern Clare have recently been undergoing population declines (partly due to roost site deterioration), which could possibly lead to the further isolation of the County Limerick horseshoe bat population (Roche et al., 2015). As the Irish horseshoe bat population already has a very low level of genetic diversity, neither subpopulation is likely to be viable in the long-term on its own, and the Limerick cluster of roosts is in a precarious position (Roche et al., 2015; Dool et al., 2016).

Therefore, while the lesser horseshoe bat population in Ireland is currently increasing, it faces a number of challenges, both immediate and longer-term. The current problems of roost deterioration or loss, and destruction of foraging and commuting habitat pose a problem in the present, but are being managed or balanced out by positive factors at least to some extent. The general trend of reafforestation in Ireland is a positive development for this species, even though the percentage of broadleaf woodland (both currently in existence and being planted) is still very low. The inclusion of a significant part of the species' range within protected areas, thus giving some protection to its habitat, is also important in this regard. The problem of roost deterioration is partially mitigated by the presence of some very high-quality roosts purposely restored or built for the use of these bats. At other roosts this problem is being kept at bay for now at least, but this requires constant effort and financial support and will continue to do so into the future. Both of these immediate problems are contributing to the longer term threat of inbreeding depression. Maintaining the genetic diversity of this species in Ireland is crucial for its survival into the future. Existing connections between subpopulations will have to be maintained to ensure continued gene flow, and connections rebuilt where these have been severed. To solve both problems, roost and habitat loss will have to be actively reversed, not just managed, in certain areas at least. This will require a national, long-term conservation plan with areas connecting subpopulations targeted for extensive habitat improvement. The measures needed to do this include the replanting of broadleaf woodland for foraging habitat, creation of habitat corridors around current barriers such as large urban areas and motorways and through areas of unsuitable habitat, and the restoration or building of potential roost sites

to encourage recolonisation of currently unoccupied areas by lesser horseshoe bats (McAney, 2014).

1.3.5 Population monitoring

Ireland's lesser horseshoe bat population has been monitored more thoroughly and over a longer period than the other Irish bat species, and the current population estimate is probably the most robust for any species. The rarity and vulnerability of this species has been the impetus for the effort to census this species as accurately as possible, and an accurate population estimate and information on population trends are important for informing conservation assessments and plans. An advantage for surveyors of this bat species is that it hangs freely from the ceiling while roosting, making it visible and easy to identify, and it is impossible to confuse with the other Irish bat species for experienced surveyors. Thus, systematic surveys of potential horseshoe bat roosts have been able to identify a large number of sites unequivocally known to be occupied by this species, allowing these to be revisited for monitoring in later years. While summer surveys usually depend on emergence counts, winter surveys can also be relatively easily carried out by counting the hibernating horseshoe bats hanging from the roost ceiling.

Annual counts of lesser horseshoe bats began with winter counts at 16 roosts in 1986, and summer counts began at 13 roosts in 1992. Both winter and summer counts have been carried out every year since then, making the lesser horseshoe bat monitoring scheme the longest running in Ireland. The number of sites monitored has increased substantially since the beginning, to 93 winter sites in 2015, and 110 summer sites in 2014. The increase in the number of monitored sites over the years is corrected for when population trends are being analysed. The total number of sites monitored are 129 winter roosts and 192 summer roosts, as some sites have not been monitored every year. Monitoring of sites has largely been carried out by NPWS and VWT staff. Data gathered from across the country was collated into a national database for the NPWS by Conor Kelleher in 2003. The management of this database was handed over to Bat Conservation Ireland in 2013, who have since subjected the data to detailed analysis of long-term population estimates and trends (Roche et al., 2015).

Winter counts are undertaken in January and February of each year, when surveyors visit hibernation roosts during frosty weather to ensure that bats are likely to be torpid. Bats are counted directly within their roosts. Summer counts are undertaken between the 23rd of May

and 7th of July each year, when surveyors count bats emerging from summer roosts at dusk (roosts are generally not entered to avoid disturbance to the bats). The two different surveys result in parallel population trend data sets which differ slightly. The summer surveys result in more bats being counted than winter surveys, with 8,727 bats counted in summer 2014 compared to 6,508 bats counted in winter 2015.

Both survey methods have shown a significant increase in the lesser horseshoe bat population since 1986/1992, estimated as a 60.7% increase based on summer count data, and a 106% increase based on winter counts (Fig. 1.6) (Roche et al., 2015). While this is clearly a positive sign for the Irish lesser horseshoe bat population, Roche et al. (2014) caution that this increase should be viewed in the context of the likely contraction in range and population caused by deforestation since the 1600s, and this population increase should really be seen as a recovery from a very low level.

Monitoring data have also been used to estimate the overall population size of this species in Ireland. Up to the mid-2000s, the lesser horseshoe bat population was estimated based on simple extrapolation from colony counts. The earliest estimate of 12,000, based on counts from 100 colonies in the mid to late 1980s, was given by O'Sullivan (1994). This was later revised downwards to 9,000-10,000 by Kelleher (2004), based on counts from 690 known roosts in the 1990s and early 2000s.

The authors of the 2007 Article 17 report attempted to take a more systematic approach to estimating the lesser horseshoe bat population (NPWS, 2007). Summer colony count data was used as the basis of this estimate; monitoring of 153 major maternity roosts in 2006 counted a total of 7565 adult bats. A further 30 known maternity roosts which were not monitored were given the average colony size of 20 individuals, bringing the total to 8165 bats in 183 maternity roosts. 25% of the animals present in maternity roosts were presumed to be males, so this number was subtracted from the total to give an estimate of 6125 female bats counted. Assuming that the entire population consists of an equal number of males and females, this number was multiplied by two to reach a final population estimate of 12,250 individuals. This method was adjusted slightly by Roche et al. (2012) as it was found that maternity roosts not regularly monitored tend to have lower populations than the overall average, and data from two consecutive years was used, partly to fill in gaps where a maternity colony may not have been counted in a particular year. Using count data from 2010-2011, the national population estimate using this revised method is now 14,010, and a retrospective figure of 13,740 for 2005-2006.

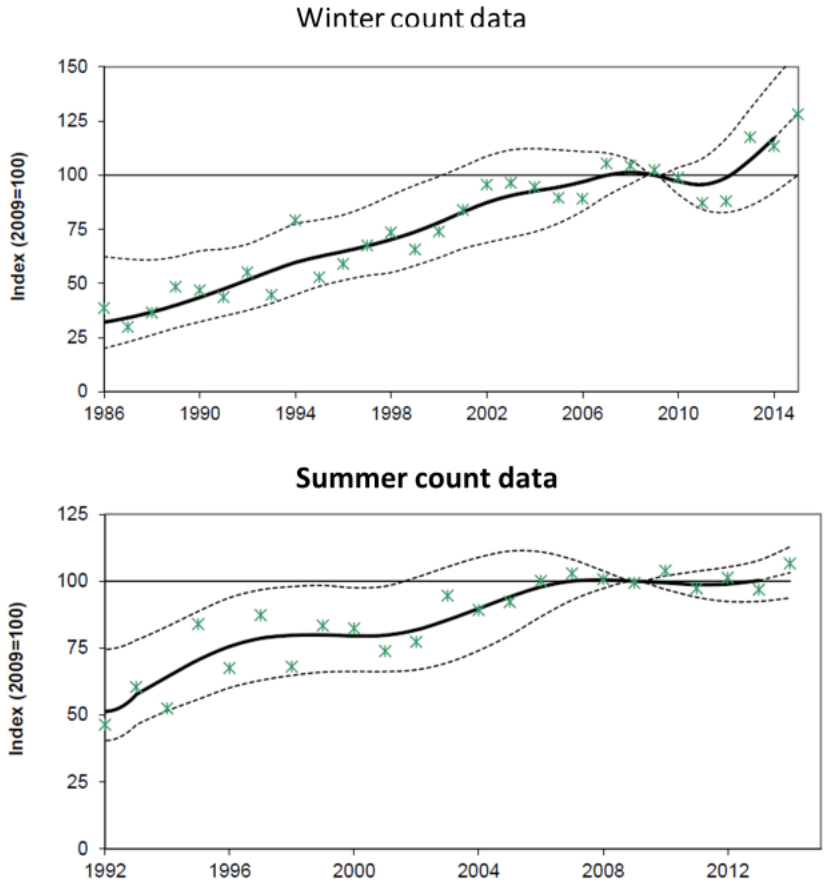

Figure 1.6: Graphs showing population trend of lesser horseshoe bat in Ireland from winter counts and summer counts, taken from Roche et al. (2015). Both graphs show a significant increase in the Irish lesser horseshoe bat population since monitoring began.

While this method may be more accurate than earlier population estimates, it is still based on two important and untested assumptions. Firstly, it assumes a 1:1 ratio of males and females in the population. Theoretically, species which reproduce sexually (including mammals) should produce equal numbers of male and female offspring. While humans and many other mammal species are indeed known to produce male and female offspring in almost equal proportions, some species are known to produce offspring in unequal sex ratios in certain circumstances (Clutton-Brock and Iason, 1986). At least one bat species, the evening bat, *Nycticeius humeralis*, is known to have a neonatal sex ratio biased towards male offspring (Bain and Humphrey, 1986). There appears to be no data on the sex ratio of lesser horseshoe bats at birth, nor on whether either sex suffers a disproportionate death rate later in life such that the sex ratio of the overall population could be skewed (NPWS, 2007). However, in an Irish study which trapped a random sample of lesser horseshoe bats, 21 out of the 39 trapped bats were female, a female-male ratio of 1.17:1, close to the theoretical 1:1 ratio (NPWS, 2007).

Secondly, this method assumes that of the adult lesser horseshoe bats present in maternity roosts in summer, 25% are male. In other words, this assumes that only one third of the adult male population is being counted, with the other two thirds of males presumably roosting in small numbers in scattered, unmonitored roosts (this population estimation method also implicitly assumes that almost the entire female lesser horseshoe bat population is being counted). This figure is based on expert opinion from lesser horseshoe bat researchers based in the UK (NPWS, 2007). Bontadina et al. (2002) trapped 90 lesser horseshoe bats out of 300 present in a maternity roost in Wales and found that 24.4% of these were male, which may form the basis of the 25% figure used in Ireland's population estimate. More recently, Zarzoso-Lacoste et al. (2018) discovered that males made up 25.8% of all bats at nineteen lesser horseshoe bat roosts in northern France, but the proportion of males at individual roosts varied from 0-50%.

Unlike the previous assumption, there is no obvious reason why 25% of lesser horseshoe bats present in maternity roosts should be males. Although there is some data to support this assumption from previous studies, the situation in Ireland may be different. If the true average proportion of males present in maternity roosts were found to be different from 25%, the current Irish population estimate could be significantly altered (a deviation of just ± 5% in the average proportion of males present in monitored roosts would alter the population estimate by almost ± 1,000 individuals). Being a rare species in Ireland, a large change of the population estimate could have an important effect on the assessment of the conservation status of the lesser horseshoe bat here, either positively or negatively.

As mentioned in the previous section of this chapter, bat droppings present a readily available source of DNA which can be non-invasively collected without the need to trap or otherwise disturb bats. The molecular techniques needed to investigate the sex ratio of lesser horseshoe bats in summer roosts, i.e. genotyping and sex typing, are well established and several studies have used DNA sex typing in particular on several other wild mammal species, such as pine marten (*Martes martes*), European wildcat (*Felis silvestris silvestris*) and a small set of North American bat species (Korstian et al., 2013; O'Neill et al., 2013; Steyer et al., 2013). These molecular techniques will be discussed in more detail in the following section. Therefore, molecular techniques have the potential to be used to address the question of lesser horseshoe bat sex ratios in summer roosts, and thus allow the national population estimate to be more accurately assessed.

1.4 Genetics and its use in wildlife studies

1.4.1 Non-invasive genetics

Non-invasive genetic sampling is described by Taberlet et al. (1999) as genetic studies of wildlife where "the source of the DNA is left behind by the animal and can be collected without having to catch or disturb the animal." This is contrasted with situations where animals must be trapped to obtain a DNA sample, which is considered invasive, i.e. disturbing the animal's normal behaviour.

This approach to collecting DNA samples from wild animals began in the early 1990s with a study of brown bears, *Ursus arctos* (Taberlet and Bouvet, 1992). Since then, its use has increased greatly as molecular techniques have improved, allowing analysis of samples containing low quantities of DNA, and varied methods of sample collection have been developed. Non-invasive sources of DNA which have been used in genetic studies of mammal species include fur, faeces, urine, saliva, spilt blood, carrion flies, parasites such as leeches and environmental DNA (Beja-Pereira et al., 2009; Calvignac-Spencer et al., 2013; Bohmann et al., 2014). Non-invasively collected DNA samples have been successfully used for a number of different purposes, including species identification, sex identification, genotyping of individuals, population estimation, and phylogeography, and methods are continually improving to allow more efficient use of such samples (Beja-Pereira et al., 2009).

Non-invasive sampling has been used in some genetic studies of bat species. Puechmaille et al. (2007) demonstrated that lesser horseshoe bat (*Rhinolophus hipposideros*) droppings yielded DNA of good enough quality to obtain genotypes from 89% of samples collected. Boston et al. (2012) also showed that droppings collected from whiskered and Natterer's bat (*Myotis mystacinus* and *M. nattereri*) roosts yielded DNA samples which produced a relatively good genotyping success rate (70-85%) compared with tissue samples from the same species (91-95%). Other studies using non-invasively collected DNA samples have also been carried out on the Indiana bat, *Myotis sodalis*, the big brown bat, *Eptesicus fuscus*, a set of 14 bat species in the Pacific North-West of the U.S.A., and the Mediterranean horseshoe bat, *Rhinolophus mehelyi* (Oyler-McCance and Fike, 2011; Puechmaille and Teeling, 2014; Vege and McCracken, 2001; Zinck et al., 2004) However, non-invasively collected DNA samples are still not commonly used in genetic studies of European bat species, which instead rely mainly

on samples invasively collected from trapped bats, mainly wing punches (wing membrane tissue).

1.4.2 Basic DNA analysis tools

1.4.2.1 Types of DNA

In animal cells, DNA is contained within two discrete areas, in the mitochondria and the nucleus (Campbell and Reece, 2002). The differing characteristics of the two types of DNA lend themselves to different purposes in wildlife genetics (Fig. 1.7).

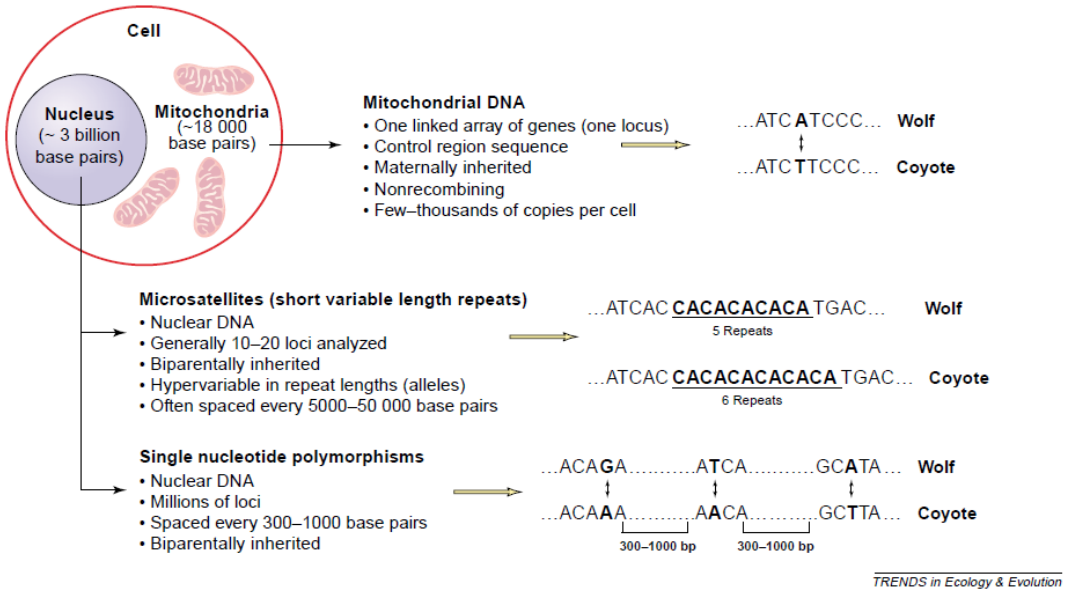

Figure 1.7: Comparison of characteristics of mitochondrial DNA and nuclear DNA markers (SNPs and microsatellites) (Morin et al., 2004).

Mitochondria contain a single copy of a relatively small amount of DNA, but due to the typically large number of mitonchondria per cell, there are numerous copies (hundreds to thousands) of this DNA in each cell. Vertebrate mitochondrial DNA (mtDNA) consists of 37 genes and a non-coding control region, and this genetic material is passed solely in the maternal line in the vast majority of species. Mitochondrial DNA has a higher rate of mutation than coding nuclear DNA, but is highly conserved within species, making it a useful for species identification. Due to the presence of multiple copies per cell, it is also useful for non-invasive

genetic studies where samples may only contain a tiny amount of DNA and may additionally be old or degraded (Allendorf and Luikart, 2007; De Young and Honeycutt, 2005).

The nucleus of the cell contains the vast majority of genetic information within the cell (billions of nucleotides), which is present in only two copies per cell and is inherited from both parents. Nuclear DNA (nDNA) contains a variety of marker types with varying mutation rates, allowing for different applications on wildlife genetics. Markers with a high mutation rate and variability such as microsatellites are useful for the identification of individuals through genotyping. In addition, a variety of genes on the sex chromosomes have been targeted to allow sex typing of individuals (Allendorf and Luikart, 2007; De Young and Honeycutt, 2005).

1.4.2.2 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is the starting point of most DNA testing methods, by amplification of the target DNA sequence which can then be analysed. Non-invasively collected samples tend to yield tiny quantities of DNA after extraction, usually in the nanogram to picogram range. To carry out PCR, a small amount of starting DNA (or template) is added to a chemical solution which contains a DNA polymerase enzyme and a pair of DNA primers, short strands of DNA which have been designed to bind to either end of the section of DNA being targeted (Rowe et al., 2017).

During the PCR process, the reaction mixture is loaded into a thermal cycler machine, which subjects it to repeated cycles of heating and cooling. Initially, the mixture is heated to 94-96°C to activate the DNA polymerase. This is followed by denaturation, during which the two strands of the sample DNA are separated. The DNA primers then anneal or bind to complementary sections on both strands of DNA, at either end of the target section of DNA. In the final step, the DNA polymerase creates a new copy of each DNA strand using each DNA primer as a starting point, thus doubling the quantity of the target DNA (Fig. 1.8). This process is repeated 40-50 times, resulting in millions of copies of the target section of DNA (Klug et al., 2010). The PCR products resulting from this reaction must then be analysed in order to determine whether the target DNA has been amplified, and numerous methods exist to do so.

PCR primers can be designed to amplify a DNA sequence specific to a target species, after which the PCR products are visualised by gel electrophoresis. This allows a positive result to be confirmed by the presence of a species-specific band or pattern of bands.

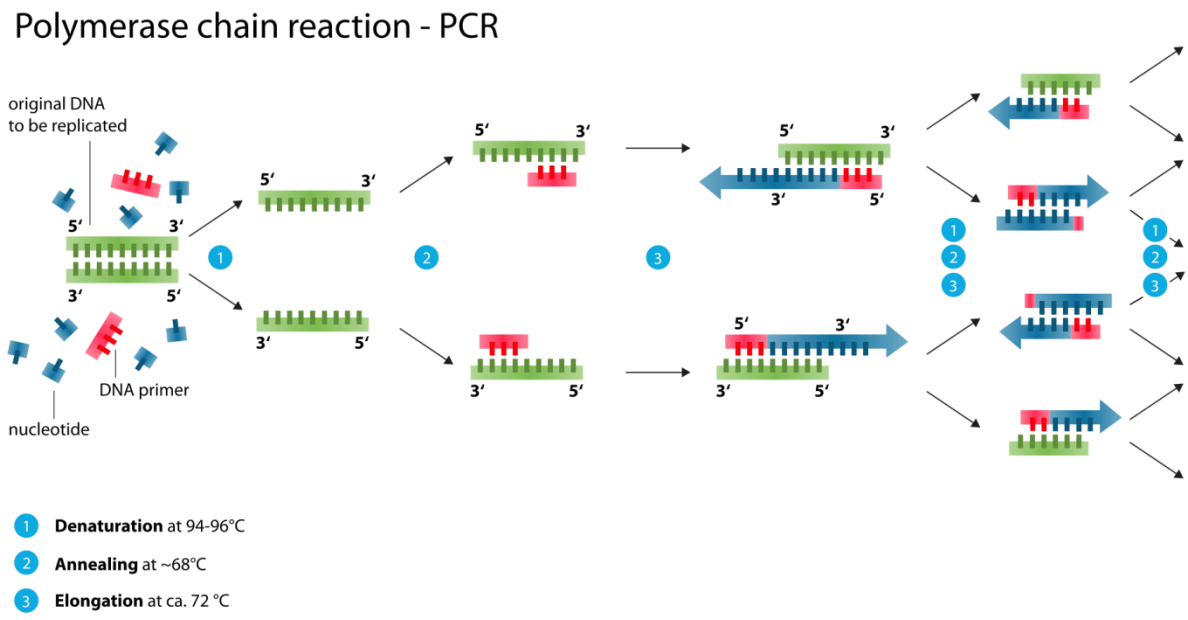

Figure 1.8: Diagram showing the process of Polymerase Chain Reaction (PCR). (Wikipedia, 2018)

This PCR method has been frequently used for species identification purposes in wildlife studies. Kanuch et al. (2007) used mitochondrial DNA typing to create species-specific DNA tests targeting specific regions of mtDNA to distinguish between the common and soprano pipistrelles, *Pipistrellus pipistrellus* and *P. pygmaeus*. PCR primers were designed based on the Cytochrome b mtDNA gene, producing bands of different sizes which could be differentiated on a gel.

Boston et al. (2011) designed a similar assay to distinguish between three morphologically similar species, *Myotis mystacinus*, *M. brandtii* and *M. alcathoe*. This study tested conventional PCR primer sets based on the ND1 gene to produce species-specific tests for these bat species, each of which only amplified and produced bands for the target species. Hamilton et al. (2015) designed species-specific assays for fifteen of the commonest bat species in the British Isles using this method.

Restriction Fragment Length Polymorphism (RFLP) analysis is another variation of PCR-based tests for species identification. Unidentified samples can be amplified via PCR using universal primers which will target the same mtDNA region across several taxa. The products of this reaction are then digested with a restriction enzyme, which splits up the DNA strand at sites where a particular combination of base pairs occur, or "restriction sites". The DNA

sequence of different target species will contain these restriction sites at different points, thus producing a distinctive pattern of differently sized bands when visualised via gel electrophoresis.

RFLP analysis has been used to distinguish between the pine marten and stone marten (*Martes martes* and *M. foina*), two closely related mustelid species (Ruiz-Gonzalez et al., 2008). The same approach was also used by Statham et al. (2005) to distinguish between five mustelid species found in Ireland: Eurasian otter *(Lutra lutra*), pine marten (*Martes martes*), European badger (*Meles meles*), stoat (*Mustela erminea*) and North American mink (*Mustela vison*).

1.4.2.3 Sequencing

DNA sequencing is a PCR-based method of identifying the species of origin of a DNA sample, whereby "universal" primers that will work for a wide range of taxa are used during the PCR process. These primers are designed to be complementary to short sequences of DNA which are conserved (i.e. are identical) across many species or even larger taxonomic groups. Such primers used for species identification will usually target a particular mtDNA gene, with commonly targeted regions including cytochrome b gene (Cyt b), cytochrome c oxidase I gene (COI), NADH dehydrogenase 1 gene (ND1), and the D-loop control region. Following PCR, the sample is analysed with a DNA Sequencer to obtain its base pair sequence, which can be compared with reference sequences from a database such as GenBank (Rowe et al., 2017).

Many genetic studies of European bats have relied on DNA sequencing, including several large-scale studies (García-Mudarra et al., 2009; Ibañez et al., 2006; Mayer and Von Helversen, 2001; Mayer et al., 2007). While most of these studies have used DNA sequencing to examine the relationships between bat species and to identify cryptic species (see Section 1.4.3) rather than for the purpose of identifying the species of origin of unidentified bat DNA samples, they have resulted in a large number of reference DNA sequences which can be presently used to assist in species identification.

Some studies of bats have used DNA sequencing for the purpose of species identification. In a study of 14 bat species in the Pacific North-West of the U.S.A., Zinck et al. (2004) designed general PCR primers targeting the 16S rRNA gene which were used to amplify DNA from faecal samples followed by sequencing of the PCR products, which allowed the reliable identification of 10 of the 14 species studied, while the remaining four species could be identified by two primer sets which amplified two pairs of closely related species. A similar

approach has been used in two studies to design primers to identify the whiskered bat and Brandt's bat (*Myotis mystacinus* and *M. brandtii),* and the Mediterranean horseshoe bat (*Rhinolophus mehelyi*), targeting the COI and Cyt b genes respectively (Boston et al., 2010; Puechmaille and Teeling, 2014).

1.4.2.4 Real-time PCR

Conventional PCR methods require a relatively large quantity of DNA template, which can lead to false negatives when using samples which contain low quantities of DNA, or where DNA is degraded and thus of low quality. Real-time PCR or quantitative PCR (qPCR) is a variant of PCR which can better deal with small quantities or poor quality DNA, as it is designed to amplify small fragments of DNA (about 50-150 bp), compared to >200 bp for conventional PCR (Matejusova et al., 2013).

There are two main chemistries used in real-time PCR, the intercalating dye (SYBR® Green 1) and fluorogenic probes (such as Taqman® MGB). Intercalating dyes will bind to any double-stranded DNA in the SYBR® Green 1 assay, whereas Taqman® probes are specific to the target DNA sequence, allowing for greater certainty in positive amplification (Bio-Rad, 2006) (Fig. 1.9).

Unlike conventional PCR, no post-PCR process is needed to confirm the presence of positive amplification in real-time PCR, such as gel electrophoresis or sequencing. Instead, real-time PCR depends on the detection of a fluorescent signal emitted by either the intercalating dye or fluorogenic probe while the PCR reaction is under way. In SYBR® Green 1 assays, the intercalating dye added to the reaction binds to any double stranded DNA present and begins to fluoresce. This fluorescent signal increases proportionally as more double-stranded copies of the targeted DNA sequence are produced during the PCR reaction. To confirm that specific amplification of the target sequence has occurred, a final step in this assay type is melt-curve analysis of the double-stranded PCR product, which can distinguish between the typically higher melting temperature (i.e. the temperature at which double-stranded DNA dissociates into two single DNA strands) of specific products against the lower temperature of non-specific products and primer-dimers (Bio-Rad, 2006).

In Taqman® probe assays, the probe has two additional molecules attached, a fluorescent dye and a quencher molecule. While both molecules are in close proximity when attached to the probe, the quencher molecule hides the light signal emitted from the fluorescent dye. When the

primers and probe have attached to the targeted DNA sequences, the polymerase enzyme begins to synthesise the new DNA strand, degrading the Taqman probe in the process as a result of its 5'-3' exonuclease activity. The degraded probe releases the fluorescent molecule, and the resulting light signal is detected, again allowing a quantitative measurement of the increase of target PCR product (Bio-Rad, 2006).

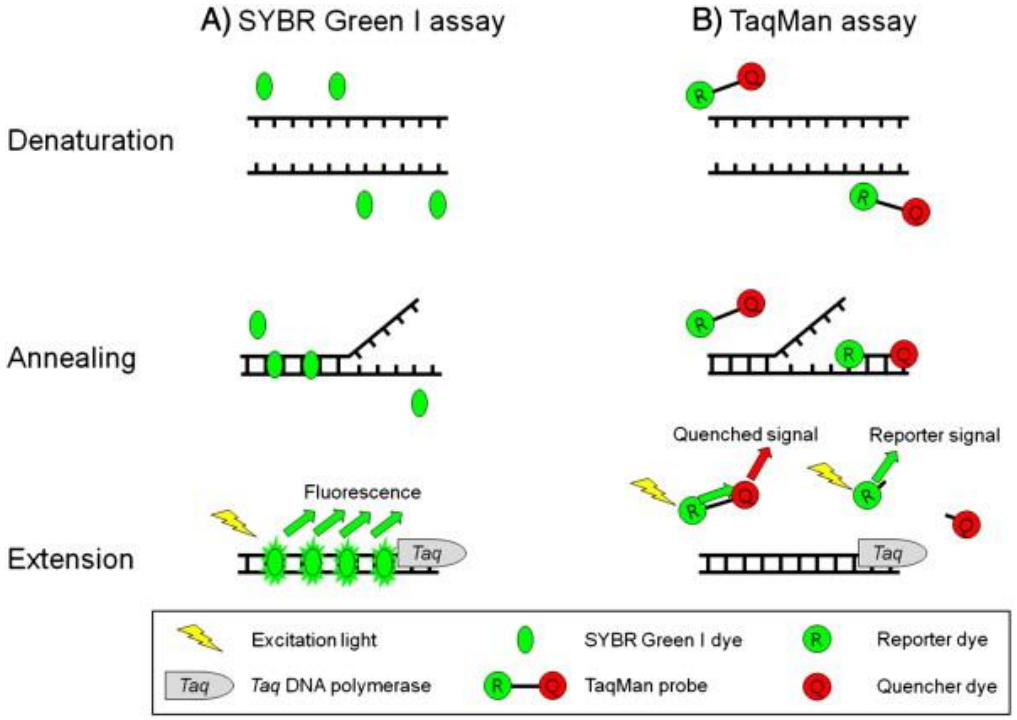

Figure 1.9: **Comparison of TaqMan and SYBR Green based fluorescence detection (Kim et al., 2013).**

This fluorescent signal accumulates as the PCR reaction progresses, and this is visualised as a graph so that DNA samples which have been successfully amplified can be identified by the exponential rise of their detected fluorescence (Figure 1.10). Once a sample's fluorescence has risen above the background fluorescence level and passed the "cycle threshold" (Ct), it is deemed to have positively amplified the target DNA of the primers used for the reaction. The value at which a DNA sample's fluorescence passes the Ct can also be used as a relative measure of the concentration of target DNA within the sample, with a lower Ct value indicating a higher concentration of DNA; therefore, a tissue DNA sample will result in a lower Ct value than a faecal DNA sample, due to the higher concentration of DNA in tissue DNA extracts. Generally, Ct values lower than 30 indicate abundant target DNA, Ct values from 31-37 indicate moderate target DNA levels, and values above 37 can be discounted as it is assumed that the quantity of target DNA is extremely low (Bio-Rad, 2006).

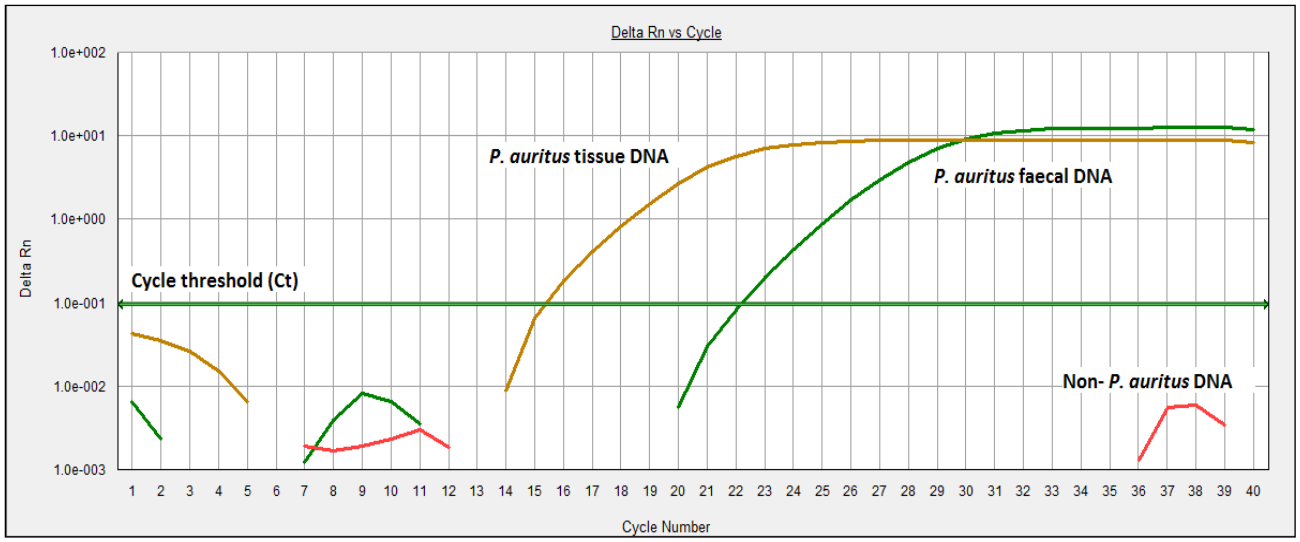

Figure 1.10: **Graph of a sample real-time PCR run from this study, showing the exponential increase of detected fluorescence of three DNA samples, indicated by different coloured lines. Two samples have risen above the cycle threshold or Ct (green horizontal line), and a non-target sample is shown whose fluorescence has not increased above the Ct.**

Real-time PCR has several advantages over both conventional PCR and sequencing, including a significant reduction in the time and cost involved in testing a DNA sample using the method (Beja-Pereira et al., 2009). In addition, real-time PCR is especially sensitive to very small quantities of DNA, such as is commonly encountered in non-invasively collected samples such as faeces, and the smaller fragments of DNA usually targeted allows even degraded DNA samples to be tested.

Real-time PCR assays have been published for a number of wild mammal species, including pine marten (*Martes martes*), red fox (*Vulpes vulpes*), Eurasian otter (*Lutra lutra*), red squirrel and North American grey squirrel (*Sciurus vulgaris* and *S. carolinensis*), roe, red and fallow deer (*Capreolus capreolus, Cervus elaphus* and *Dama dama*) and a set of five small mammal species present in the British Isles: wood mouse, *Apodemus sylvaticus*; bank vole, *Myodes glareolus*; water shrew, *Neomys fodiens*; pygmy shrew, *Sorex minutus*; and common shrew, *S. araneus* (Fajardo et al., 2008; Moran et al., 2008; O'Meara et al., 2012; O'Neill et al., 2013; O'Reilly et al., 2007).

The ability of real-time PCR to provide a relative measure of the concentration of target DNA in a sample is also useful for genetic studies using non-invasively collected material by providing information on the potential quality of individual samples for more detailed genetic analysis which would require high-quality DNA samples. For example, O'Neill et al. (2013)

used real-time PCR to test non-invasively collected faeces from Eurasian otters (*Lutra lutra*), using both mtDNA and nDNA markers. O'Neill et al. (2013) subsequently selected those samples with the lowest mtDNA Ct values (and hence with the highest concentrations of DNA) for genotyping to identify individual otters, which ideally requires samples with a high concentration of nuclear DNA. This allowed the best quality samples to be selected for detailed analysis and avoided wasting resources on poor-quality samples unlikely to produce usable genetic data.

1.4.3 Cryptic bat species

Many of the genetic studies carried out on European bat species since the mid-1990s have focused on their phylogeny and the identification of cryptic bat species. A knowledge of the potential existence of cryptic lineages within species of interest is required when the design of species identification assays is being considered.

Genetic studies of European bats have succeeded in identifying several previously unknown cryptic species since the 1990s. These include the soprano pipistrelle (*Pipistrellus pygmaeus*), as mentioned above; Hanak's pipistrelle (*Pipistrellus hanaki*), distinguished from the soprano pipistrelle; the Alpine long-eared bat (*Plecotus macrobullaris*) and Sardinian long-eared bat (*Plecotus sardus*), previously identified as the brown long-eared bat (*Plecotus auritus*); the Balkan long-eared bat (*Plecotus kolombatovici*), once identified as the grey long-eared bat (*Plecotus austriacus*); the Alcathoe bat (*Myotis alcathoe*), previously considered to be whiskered bats (*Myotis mystacinus*); and Escalera's bat (*Myotis escalerai*), previously included in Natterer's bat (*Myotis nattereri*) (Helversen et al., 2001; Kiefer et al., 2002; Mucedda et al., 2002; Benda et al., 2004; Ibañez et al., 2006; Spitzenberger et al., 2006). Of the species resident in the British Isles, some have been shown to be relatively genetically homogenous, while others appear to be highly genetically diverse across Europe and are still being investigated to identify new cryptic species or distinct subspecies.

Of the British bat species, the common noctule (*Nyctalus noctula*), greater horseshoe bat (*Rhinolophus ferrumequinum*), grey long-eared bat (*Plecotus austriacus*), Bechstein's bat (*Myotis bechsteinii*) and the Barbastelle bat (*Barbastellus barbastella*) do not appear to show sufficient population structure or highly distinct lineages within Europe that would indicate the presence of cryptic species (Petit et al., 1999; Kerth et al., 2008; Flanders et al., 2009; Rebelo et al., 2012; Razgour et al., 2013).

Three species have been found to consist of distinct lineages on some isolated islands in Europe. An as yet undescribed cryptic species previously identified as the common pipistrelle (*Pipistrellus pipistrellus*) has been found to occur in Corsica and Sicily, and a divergent lineage of the soprano pipistrelle (*P. pygmaeus*) in Cyprus is now treated as a subspecies, *P. p. cyprius* (Hulva et al. 2007; Hulva et al., 2010). Leisler's bat (*Nyctalus leisleri*) has been found to be very similar genetically to the Azores noctule (*N. azoreum*), although both species are isolated geographically and are behaviourally and morphologically distinct (Boston et al., 2015). A lack of current gene flow between *N. leisleri* and *N. azoreum* has been used by some authors to justify distinct species status for *N. azoreum* (Salgueiro et al., 2010). However, Boston et al. (2015) discovered that both species share almost identical mtDNA haplotypes, split into two main lineages. The British Isles form a zone of intermixture between these western (Azores-British) and Eastern (mainland Europe) lineages, which led Boston et al. (2015) to argue that *N. azoreum* should be considered a subspecies of *N. leisleri*. Other than these island populations, *P. pipistrellus*, *P. pygmaeus* and *N. leisleri* have all been found to be relatively genetically homogenous across Europe (Hulva et al., 2010; Boston et al., 2015).

Three bat species have been shown to be genetically diverse enough to strongly suggest that further cryptic or distinct subspecies remain to be found. Escalera's bat (*Myotis escalerai*) was recently discovered by genetic analysis of bats identified as Natterer's bats (*M. nattereri*) from Iberia. Further genetic analysis of Natterer's bats has revealed another probable cryptic species (which has yet to be formally scientifically described), which is present in northern Iberia, southern France, and Italy, with *M. nattereri sensu stricto* apparently restricted to northern and central Europe and the Balkans (Salicini et al., 2013). Spitzenberger et al. (2006) identified four highly distinct lineages within the brown long-eared bat (*Plecotus auritus*), present in (a) the Caucasus, (b) Eastern and South-Eastern Europe, and the eastern edge of the Alps in eastern Austria and north-east Italy, (c) western Austria and Italy, and (d) Iberia. The Iberian brown long-eared bats have previously been described as a distinct subspecies, *P. a. begognae*, but no further studies have been carried out to clarify the potential for cryptic species within the brown long-eared bat in the rest of Europe (Juste et al., 2004; Mayer et al., 2007). Dool et al. (2013) carried out a large-scale genetic study of the lesser horseshoe bat (*Rhinolophus hipposideros*) and did not find evidence of cryptic species. However, several karyotypic variants (i.e. with differing numbers of chromosomes) of this species occur within Europe, and several authors have suggested that gene flow between these variants should be investigated to examine

whether these possibly represent different cryptic species (Puerma et al., 2008; Dool et al., 2013; Volleth et al., 2013).

Two species show unexpectedly close genetic relationships to other morphologically distinct bat species across Europe. The greater mouse-eared bat (*Myotis myotis*) and lesser mouse-eared bat (*Myotis blythii*) share the same mtDNA haplotypes in southern Europe where they co-exist. This is thought to have resulted from interbreeding between the two species, with mtDNA haplotypes from *M. myotis* introgressing into *M. blythii* (Mayer & Von Helversen, 2001; Berthier et al., 2006). The serotine bat (*Eptesicus serotinus*) was found by Mayer and Von Helversen (2001) to share very similar mtDNA haplotypes with the northern bat (*E. nilssonii*), with *E. serotinus* mtDNA haplotypes being nested within *E. nilssonii* haplotypes in a cladogram. The authors suggest that this could be explained by a rapid speciation event in the relatively recent past which resulted in the appearance of *E. serotinus*. In the case of both species pairs, it would be difficult to design species identification assays to differentiate between each based on mtDNA genes, as they share highly similar or identical mtDNA sequences.

For the remaining British bat species, there is at best a patchy picture of their genetic diversity across Europe so far. Simoes et al. (2007) argue that some Daubenton's bat (*Myotis daubentonii*) populations in Iberia should be considered a distinct subspecies, *M. d. nathalinae*, based on sequence differences in mtDNA genes between this putative subspecies and "*M. d. daubentonii*". However, the level of mtDNA differentiation between these putative subspecies was similar to that seen in *M. daubentonii* individuals between different populations across central and western Europe in other studies, some of which have also shown extensive interbreeding between these populations using microsatellite marker analysis (Mayer and Von Helversen, 2001; Ibañez et al., 2006; Ngamprasertwong et al., 2008; Atterby et al., 2009). All of these studies are somewhat limited in geographical scope and/or number of sampled individuals, but the overall indication is that no cryptic species await discovery at least in central and western Europe. Similarly, the whiskered bat, *M. mystacinus sensu stricto* (i.e. excluding Brandt's and Alcathoe bats) has not been studied in as great detail as other European bat species, but the available information suggests that a distinct subspecies, *M. m. bulgaricus* exists in the Balkan peninsula, with populations in central and western Europe being more homogenous (Mayer and Von Helversen, 2001; Ibañez et al., 2006; Mayer et al., 2007). No genetic studies have focused in detail on the Alcathoe bat (*M. alcathoe*), Brandt's bat (*M. brandtii*), or Nathusius' pipistrelle (*P. nathusii*). Large-scale genetic studies of European bats

did not indicate the presence of distinct subspecies or cryptic species, but the number of samples obtained was very small, so the conclusions that can be drawn about these species is limited (Mayer and Von Helversen, 2001; Ibañez et al., 2006; Mayer et al., 2007).

1.4.4 Sex typing

Determining the sex of individual animals in a population is a common question addressed in molecular ecology studies, which can provide much information on a species' ecology and may be crucial to informing conservation measures for a particular species. While this has traditionally depended on live trapping animals and physically checking their sex, numerous molecular ecology studies have addressed this question by developing DNA based sex typing assays and applying them to non-invasively collected DNA samples (Beja-Pereira et al., 2009).

Molecular sex determination of mammals focuses on the sex chromosomes (X and Y), specifically by using PCR to amplify DNA sequences which are either specific to the Y chromosome (thus identifying males only), or by amplifying homologous regions of both the X and Y chromosomes, allowing both sexes to be positively identified (Shaw et al., 2003).

1.4.4.1 Zinc Finger (ZF) Genes

Zinc Finger (ZF) genes are present on both the X and Y chromosomes, termed the ZFX and ZFY genes, respectively. These are homologous regions, meaning that their DNA sequences are highly similar to each other, and PCR amplification of these regions results in sex specific PCR products (Shaw et al., 2003).

Cathey et al. (1998) designed primers to target a section of both the ZFX and ZFY genes of several deer species. These primers produce PCR products of slightly different sizes from the two genes, resulting in different banding patterns for female and male individuals (a single band, and a pair of bands, respectively) when visualised using gel electrophoresis. Shaw et al. (2003) demonstrated the use of these primers across a range of mammalian taxa, including cetaceans, carnivores and artiodactyls. Korstian et al. (2013) used the same primers to design taxon-specific sex typing primers for five North American bat species (including four Vespertilionid species and one Molossid species), again producing a single band for females and two bands for males when visualised on a gel.

Aasen and Medrano (1990) used two different approaches to using the ZF genes for sex typing. Primers were designed to amplify almost identically sized section of the ZFX/ZFY genes, and the PCR products were subsequently analysed either by RFLP analysis, or by sequencing. When sequenced, the female individuals showed only single peaks in the sequence chromatogram, while males displayed chromatograms with double peaks at a number of points in the DNA sequence. Statham et al. (2007) used this method to sex type five mustelid species present in Ireland: Eurasian otter *(Lutra lutra*), pine marten (*Martes martes*), European badger (*Meles meles*), stoat (*Mustela erminea*) and North American mink (*Mustela vison*).

Mullins et al. (2010) designed real-time PCR sex typing assays targeting fragments of the ZFX and ZFY genes to sex type pine marten (*Martes martes*), and the same approach was used by O'Neill et al. (2013) to for a sex typing assay for the Eurasian otter (*Lutra lutra*).

1.4.4.2 Sex-determining Region Y (SRY) Gene

The sex-determining region Y (or SRY) gene, also known as the testis-determining factor (TDF), is only found on the Y chromosome, and is responsible for the development of male embryos in mammals (Sanchez et al., 1996). As it is specific to males, the SRY gene has been used in many studies where sex typing is required, on a wide range of mammalian taxa including primates, rodents, insectivores, carnivores, and artiodactyls (Dallas et al., 2000; Pomp et al., 1995; Reed et al., 1997; Sanchez et al., 1996).

Bullejos et al. (2000) demonstrated that sex-typing primers based on the SRY gene could distinguish male bats from four megabat and four microbat species, producing a single band in gel electrophoresis from DNA from male bats. Similarly, Bryja and Konecny (2003) demonstrated that sex-typing primers based on the SRY gene developed by Sánchez et al. (1996) could clearly distinguish between male and female *Myotis myotis*, a European Vespertilionid bat species, as well as several European rodent species.

A drawback of using primers to target the SRY gene is the risk of false negatives. Due to the fact that only a single copy of the SRY gene exists per cell (compared with the large number of copies of mtDNA genes), poor DNA samples such as those potentially obtained via non-invasive sampling may be incorrectly labelled as female due to a lack of amplification of the SRY gene. To compensate for this, another target gene present in both male and female animals is used as an internal control, the ZFX gene often being used for this purpose. The use of such an internal control was demonstrated by Pomp et al. (1995), who used a primer set targeting

the SRY gene in tandem with primers targeting the ZFX gene as an internal control in order to determine the sex of pig embryos.

1.4.4.3 DEAD box (DB) Genes

Homologous DEAD box genes are located on the X and Y chromosomes, named DBX and DBY respectively, and are involved in the synthesis of RNA helicase enzymes. A number of primers targeting conserved regions of the DBY gene were designed by Hellborg and Ellegren (2003). These have been found to be useful for identifying males across a wide variety of taxa including primates, carnivores, artiodactyls, rodents, cetaceans and a single bat species (*Myotis daubentonii*), among others (Hellborg and Ellegren, 2003).

The primers designed by Hellborg and Ellegren have since been used by other studies of bat species, including members of the family Emballonuridae, and a set of eight Neotropical bat species, albeit for the purposes of phylogenetics (Clare, 2011; Lim et al., 2008). Dool (2010) also used Hellborg and Ellegren's DBY3 primer set to identify male *R. hipposideros* individuals from tissue samples of unknown sex.

1.4.4.4 Sex typing of non-invasively collected DNA samples

One difficulty of molecular sex typing is that the sex chromosomes form part of the nuclear DNA of mammalian cells, and therefore only a single pair of sex chromosomes is present in each cell. The quantity of target DNA for sex typing assays is already very low compared to mtDNA even in good quality tissue samples, and the quantity obtainable from non-invasively collected samples is far lower still, increasing the difficulty of sex typing non-invasive samples. Nevertheless, several studies have successfully sex typed non-invasively collected samples such as seal faeces, otter spraints, wolf fur, and remains of bats killed by wind turbines (Korstian et al., 2013; Mucci and Randi, 2007; Reed et al., 1997; Sastre et al., 2009).

While the studies mentioned above used conventional PCR primers to produce DNA fragments which could be visualised via gel electrophoresis, sex-typing primers for other mammal species have been designed using real-time PCR, such as for pine marten (Mullins et al., 2010) and otter (O'Neill et al., 2013). The sensitivity of real-time PCR to extremely small quantities of target DNA makes it especially suitable for sex typing of non-invasive DNA samples.

1.4.5 Individual identification

DNA analysis for the identification of individual animals (genotyping), including from non-invasive samples, has been used in studies on a broad range of species since the 1990s (Rowe et al., 2017; Beja-Pereira et al., 2009). Microsatellites are the most commonly used type of genetic marker currently used for genotyping. Distributed throughout the nDNA of the cell, microsatellites consist of regions of short motifs of 1-6 bp, which are typically repeated 5-40 times. Due to this repetitive character, they are sometimes also known as simple sequence repeats (SSRs) or short tandem repeats (STRs). Microsatellites are analysed by initial amplification via PCR using suitable primers, usually creating products 100-300 bp in length, followed by fragment analysis (essentially an advanced form of gel electrophoresis) to ascertain their length in base pairs (Selkoe and Toonen, 2006).

Microsatellites in an individual are inherited equally from both parents, unlike mtDNA. They are commonly located within non-coding regions of DNA, meaning that they can be prone to frequent mutations (usually by an increase or decrease in the number of motif repeats) and thus can be highly variable, with several alleles of different length. This variation in length and their tendency to mutate make them suitable for identifying individuals, as a panel of several microsatellites will produce a unique genetic "fingerprint" for an individual animal due to the combination of microsatellite alleles of differing sizes. Their inheritance from both parents and mutability also make them very useful for examining gene flow within and between populations, by measuring the different patterns of microsatellite alleles. Their rapid mutation rate makes them useful for studying the recent history of a population (Schlötterer, 2004).

Microsatellites for use in genotyping *R. hipposideros* and the closely related *R. ferrumequinum* are already available. Puechmaille et al. (2005) described a panel of 14 polymorphic microsatellite markers for *R. hipposideros*, with the number of alleles for each microsatellite ranging from two to nine. The authors used eight of these microsatellites in a multiplex to genotype 91 individual bats in a single roost in France from non-invasively collected faecal pellets. Puechmaille and Petit (2007) carried out a larger non-invasive survey of three *R. hipposideros* roosts in northern France, obtaining droppings in two once-yearly sampling sessions, collecting approximately three times as many droppings as the number of bats that had been visually observed inside each roost. Using the same microsatellite multiplex as Puechmaille et al. (2005), they genotyped 165 individuals from the three roosts. Although the number of individuals genotyped from each roost was consistently lower than the number of bats visually observed, a high proportion of the bat population at each site was detected from

DNA samples collected during each survey. Another six microsatellites have been designed for *R. hipposideros* by Dool et al. (2013) and Struebig et al. (2011) bringing the total number of currently available microsatellite markers to 20.

1.5 Objectives of this PhD thesis

There are two overall aims of this project, both based on non-invasive sampling: firstly, to design DNA based species identification tests for the British bat fauna; and secondly, to assess the sex ratio of the lesser horseshoe bat at summer roosts in order to improve population estimates for this species in Ireland. More specifically, the main objectives of this thesis are as follows:

- Develop real-time PCR assays for the identification of eighteen bat species known to occur in the British Isles.
- Carry out a field survey of potential bat roost sites in Co. Waterford in Ireland to collect non-invasive bat DNA samples and identify the bat species present at these roosts using the real-time PCR assays.
- Develop a sex-typing assay for the lesser horseshoe bat and optimise a set of microsatellite markers for genotyping of the lesser horseshoe bat faecal DNA.
- Use the previously designed and optimised species, sex and genotyping assays to study lesser horseshoe bat populations at a number of summer roosts to ascertain the sex ratio of adult bats present, for comparison with the assumed sex ratio of males currently used in population estimates of this species.
- Use microsatellite data obtained on lesser horseshoe bats in the previous objective to examine whether isolated subpopulations exist in the north of this species' range in Ireland.

Chapter 2

Development of species-specific real-time PCR primers for bat species of the British Isles

2.1 Introduction

Determining the distribution of a particular species is a common aim in wildlife biology studies, and non-invasive sampling coupled with genetic analysis is widely applied to such studies of wild mammal species, especially where there may be a need to differentiate between closely related or difficult to distinguish species (Beja-Pereira et al., 2009). Bat droppings are readily available at roost sites, and have been shown to be a source of high-quality DNA samples (Vege and McCracken, 2001; Puechmaille et al., 2007; Boston et al., 2012), with the potential to be used in species distribution surveys or to distinguish between cryptic species which may be difficult to tell apart with other survey methods.

However, non-invasive genetics has been used in relatively few studies to examine the distribution of bat species or for confirmation of the identity of specimens of cryptic species. Zinck et al. (2004) published a set of PCR assays for 14 North American bat species and described their use in non-invasive distribution surveys for these species in north-western USA. Puechmaille and Teeling (2014) also used non-invasive genetic methods in a survey of a hibernation roost in France, to distinguish between the rare bat species *Rhinolophus mehelyi* and the visually very similar but more common species *R. euryale*.

PCR-based species identification assays have been previously designed for bat species resident in the Great Britain and Ireland (Boston et al., 2011; Hamilton et al., 2015). However, no comprehensive set of species identification assays for all of the resident bat species present here has yet been published: Boston et al. (2011) presented a set of PCR primers to identify three cryptic *Myotis* species (*M. mystacinus*, *M. brandtii* and *M. alcathoe*), while Hamilton et al. (2015) published a set of primers for 15 of the British bat species, but did not include *M. brandtii*, *M. alcathoe* and *M. myotis*. In addition, while these studies described their assays as "rapid", both methods involve a significant amount of post-PCR processing, either gel electrophoresis (Boston et al., 2011) or PCR fragment size analysis using a DNA sequencer (Hamilton et al., 2015). This post-PCR processing adds further cost, work and the potential to introduce errors, which can be avoided by the use of real-time PCR, which has no post-PCR processing steps and also has the advantage of being highly sensitive to the to the very small quantities of DNA found in non-invasively collected samples (O'Neill et al., 2013).

The usefulness of real-time PCR for this purpose has been demonstrated by its application in numerous non-invasive genetic studies of mammal species resident in the British Isles,

including pine marten, *Martes martes* (O'Reilly et al., 2007; Mullins et al., 2010; O'Mahony et al., 2015; O'Mahony et al., 2017); otter, *Lutra lutra* (O'Neill et al., 2013; White et al., 2013); red squirrel, *Sciurus vulgaris* (O'Meara et al., 2018); and small mammals (Moran et al., 2008; O'Meara et al., 2014). The development of a full set of species identification assays for all British and Irish bat species based on real-time PCR would provide a valuable new tool for bat surveyors, both for identifying specimens belonging to cryptic species (either from tissue or faecal DNA samples), and for application in non-invasive surveys, as well as significantly reducing the amount of laboratory analysis needed compared to the existing methods.

The aim of this chapter is to describe the development of a set of real-time PCR assays for the identification of the 18 resident bat species of the British Isles, *Barbastella barbastellus, Eptesicus serotinus, Myotis alcathoe, M. bechsteinii, M. brandtii, M. daubentonii, M. mystacinus, M. nattereri, M. myotis, Nyctalus leisleri, N. noctula, Pipistrellus nathusii, P. pipistrellus, P. pygmaeus, Plecotus auritus, Pl. austriacus, Rhinolophus ferrumequinum* and *R. hipposideros*. The use of these assays in a non-invasive survey of potential bat roost sites in Ireland will also be demonstrated.

2.2 Materials and Methods

2.2.1 Primer Design and Validation

2.2.1.1 Reference Sample Collection

A collection of reference DNA samples was assembled for all 18 bat species which were to be included in this study. In total 81 samples were obtained, including both tissue and dropping samples (Table 2.1), all of which were stored at -20°C.

Tissue samples were obtained from dead bats which were found in the field in County Waterford, or were obtained from the collections of other bat surveyors and researchers. Droppings for use as reference samples were collected either directly from bats which had been trapped and identified in the hand by experienced bat workers, or obtained from a store of bat droppings already available at WIT which had been previously DNA sequenced to identify the species of origin.

Table 2.1: Table of reference samples obtained for each British bat species.

Species	No. of tissue samples	No. of dropping samples	Total
Barbastella barbastellus	-	3	3
Eptesicus serotinus	-	4	4
Myotis alcathoe	-	2	2
M. bechsteinii	4	-	4
M. brandtii	-	3	3
M. daubentonii	1	2	3
M. myotis	1	-	1
M. mystacinus	1	3	4
M. nattereri	1	2	3
Nyctalus leisleri	1	1	2
N. noctula	-	2	2
Pipistrellus nathusii	1	3	4
P. pipistrellus	2	2	4
P. pygmaeus	15	8	23
Plecotus auritus	3	2	5
Pl. austriacus	-	3	3
Rhinolophus ferrumequinum	-	3	3
R. hipposideros	6	2	8
Total	36	54	81

2.2.1.2 DNA Extraction

For tissue samples, a small section (approx. 5 mm diameter) of wing tissue was cut off from each bat being sampled with scissors and tweezers, which were alcohol flamed between samples to prevent cross-contamination. The tissue DNA was then extracted using the ZR Genomic DNA™- Tissue MicroPrep (Zymo Research, cat. no. D3051) according to the Solid Tissue protocol, with Zymo-Spin™ II columns (Zymo research, cat. no. 3041).

Once extracted, the tissue sample concentrations were measured using a Thermo Scientific Nanodrop™ 1000 Spectrophotometer (Appendix 1). Tissue concentration measurements allowed for accurate dilution of working samples and creation of serial dilutions for the measurement of standard curves for each species primer set.

For bat faecal pellet samples, a single bat faecal pellet was transferred to 500 µl of Stool Transport and Recovery (STAR) Buffer (Roche, cat. no. 03335208001), vortexed to mix and allowed to stand at room temperature for ≥30 min. The sample was then centrifuged at 1000 g

for 60 s and 150 µl of supernatant was removed for DNA isolation using the Solid Tissue protocol as above, starting at step 3. All DNA extracts were stored at -20°C.

2.2.1.3 Primer design

Due to its interspecific variability the mtDNA Cytochrome b gene has been commonly used in phylogeographic studies of mammal species, including numerous European bat species, and has contributed to the identification of several cryptic species. As a result, Cyt b sequences for many European bat species are available on GenBank (NCBI) database (Clark et al., 2016). Therefore, this gene was selected as the target for real-time PCR primers for this study as sequences were available for all of the species of interest, and would make direct comparison between all 18 species more straightforward. Sequences of the Cyt b gene from each of the bat species in this study were downloaded from GenBank (Appendix 2).

These sequences were aligned in MEGA 6.0 using the Clustal W algorithm. Species-specific sites were identified by eye and targeted as potentially useful regions for primer design. Real-time PCR primers were designed using Primer Express v2.0 (Applied Biosystems). Primer sets were designed to target species-specific nucleotide polymorphisms at the 3' ends of both primers. The design parameters used included a melting temperature of between 58°C and 60°C, primer length of between 20 and 30 bp, a GC content of between 45% and 55% and the production of an amplicon between 70 and 100 bp long. Primer sets were checked using BLAST searches to ensure that they were specific to the target species (Altschul et al., 1997). Primers were ordered from Eurofins (Germany) in a lyophilised state and were resuspended in sterile water to a stock concentration of 100 pmol/µl. Aliquots were then diluted with H_2O to a working concentration of 5 pmol/µl (5 µM) each of both forward and reverse primers for use in the real-time PCR reactions.

2.2.1.4 Primer specificity

To ensure that each primer set was species-specific, the primers were used to test the reference material of known species. Samples were tested by creating a mixture of 5 µl of Faststart Universal SYBR Green Master (ROX) (Roche, cat no. 04913914001), 0.4 µl of each primer mix, with 1 µl of DNA template, and H_2O to a total volume of 10 µl per well. Tissue samples were diluted to a standard 4 ng/µl for this purpose. Negative controls contained molecular grade H_2O instead of DNA. As it was not possible to accurately measure the quantity of target

DNA in faecal samples (due to the presence of bacterial and prey DNA), and target DNA quantity was likely to be far lower in any case, these samples were not diluted for testing.

The samples to be tested were loaded into a MicroAmp Optical 96-well reaction plate (Applied Biosystems, cat. no. N8010560) and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, cat. no. 4311971). The PCR reaction was carried out using an Applied Biosystems 7300 Real-Time PCR System with a default profile of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. During this process, fluorescence was measured and recorded at the end of each cycle.

A final dissociation step of 15s at 95°C, 30s at 60°C and 15s at 95°C was used for melt curve analysis to confirm specific amplification. During this step, the temperature of the PCR plate is gradually increased and the decrease in SYBR Green fluorescence measured as the PCR product melts (i.e. the double-stranded DNA dissociates). A dissociation curve is produced which identifies the melting temperature of the target amplicon.

All primer sets were tested to examine their specificity using the reference samples, which were divided into three sets: tissue samples, droppings of known species and droppings from known species roosts. For each reference sample set, each sample was tested using all primer sets. The lowest resulting Cycle threshold (Ct) value for each sample was used to assign a species identification result, and this was cross-referenced with the known species of each sample to check if the correct species had amplified. Samples showing positive amplification with a Ct value greater than 30 were disregarded, as it was considered that such results were highly likely to be due to non-specific amplification, i.e. a false positive result.

2.2.1.5 Primer sensitivity and amplification efficiency

To test the amplification efficiency and sensitivity of the primers, serial dilutions of tissue DNA (4 ng/µl - 4 X 10^{-6} ng/µl) were amplified for each species where possible, using the same real-time PCR method as above. All tissue DNA extracts were diluted to a standard 4 ng/µl.

The Ct values which resulted from testing of serial dilutions were used to create a standard curve, plotting Ct values against the logarithm of DNA concentration. The standard curve was used to estimate the R^2, showing the sensitivity of the primers, and the gradient of the curve, which gives a measure of the amplification efficiency of the primers.

2.2.2 Field Survey of Bat Roosts

2.2.2.1 Study area and sample collection

Potential bat roost sites across County Waterford were surveyed from 2011-2014. The overall survey was composed of several smaller surveys of distinct roost types, which were investigated at different times of the year according to their different potential uses by bats, e.g. summer surveys for potential maternity sites, and late winter surveys for potential hibernation sites.

Roost surveys consisted of a thorough search of the site for signs of the presence of bats. Indicators of the presence of bats included bat droppings, urine stains, prey remains (usually moth or butterfly wings), bat carcasses, and in some cases live bats were found. Signs of the presence of bats, or a lack thereof, for each site surveyed were noted, and any live bats which could be identified were also recorded. Bat carcasses and droppings, where available, were collected from each site for DNA testing later. Night-time bat detector surveys were also carried out at a subset of the roosts.

Churches in County Waterford were identified as a recognisable roost site type which could be systematically surveyed, as had been done previously in England and Wales (Sargent, 1995). One previous study in Ireland had surveyed a selection of Church of Ireland (C of I) churches across several counties surrounding Dublin to identify new roost sites (Roche, 1998). In this study, all church buildings within County Waterford (almost all either Roman Catholic or Church of Ireland) were considered for surveying for signs of the presence of bats in the summer months (i.e. May to September), as these were most likely to be used by bats as maternity sites. In total, 103 church buildings currently in use were identified (although some had been converted for different uses), of which 73 were surveyed. Five ruined church buildings were also included in the survey.

Underground sites such as caves, tunnels, mines, cellars and ice houses are known to be used by certain bat species as winter hibernation roosts, and some are used as "swarming sites" for mating in autumn (Glover and Altringham, 2008). In total, nine underground sites in County Waterford were selected for survey, including seven natural limestone caves, a disused railway tunnel and a mill race tunnel located beneath the ruin of a 19th-Century saw mill. The sites were surveyed in February-March of 2013 and 2014 to investigate the possibility of use by bats as hibernation sites. A subset of the sites which showed potential for use as swarming sites were also checked for signs of bat activity from July to September 2014.

Eighteen Schwegler 2F woodcrete bat boxes were erected on trees in pairs at two separate sites in County Waterford in September 2013. Twelve boxes were placed in an area of mixed river-side woodland immediately to the north of the town of Lismore, and six were placed along treelines and in mixed woodland around the edges of the village of Cheekpoint. After being erected, these were checked in May and September 2014 to look for signs of bat occupation.

In the course of surveys for bat and other mammal species, signs of bat occupation were found in a number of other sites of varying types, including bridges, houses (including several large manor houses), farm buildings, garages, disused schools and gate lodges.

To increase the number of samples from unknown roosts available for real-time PCR testing, several sets of bat dropping samples were donated by bat surveyors carrying out similar surveys in three counties outside of Waterford, in Galway, Kildare and Wexford. Samples collected as part of church bat surveys in counties Kildare and Wexford were obtained, as well as samples from two bat box schemes located in Kildare and Galway (all consisting of Schwegler 2F woodcrete bat boxes). A small number of samples from "other" roosts from all three counties were also obtained.

2.2.2.2 DNA testing

Where bat faecal samples were collected, DNA was extracted and tested as described in section 2.2.1. The majority of DNA extracts were from a single bat dropping from a sample. However, a subset of samples were selected to extract DNA using several bat droppings from the same site. In some cases this was unavoidable, as with samples collected from bat boxes where bat droppings had often degraded into a powdery mass such that it was difficult to select a single dropping. In samples where multiple droppings had been collected and it was thought possible that several bat species could co-exist at the same roost site, DNA was extracted from two to four bat droppings simultaneously, using the same protocol as for single-dropping DNA extractions. Where possible, droppings for DNA extraction were selected to pick out a variety of sizes and shapes when obvious differences between droppings were seen, potentially indicating different species of origin.

Once extracted, the DNA samples were tested using the set of real-time PCR primers for all bat species recorded in Ireland, i.e. *P. pipistrellus*, *P. pygmaeus*, *P. nathusii*, *N. leisleri*, *P. auritus*, *M. daubentonii*, *M. nattereri*, *M. mystacinus*, *M. brandtii*, *R. hipposideros*, and *R. ferrumequinum*.

After testing for bat species had been carried out, DNA samples which remained unidentified were also tested for non-target species to examine whether misidentification of bat droppings during sample collection may have occurred. Samples were tested for the Irish small mammal species most likely to be present in the areas surveyed: wood mouse (*Apodemus sylvaticus*), pygmy shrew (*Sorex minutus*), bank vole (*Myodes glareolus*), and brown rat (*Rattus norvegicus*). Real-time PCR primers designed for these species by Moran et al. (2008) and Moran (2009) were used.

2.3 Results

2.3.1 Primer Design and Validation

The primer sets designed for each bat species are listed in Table 2.2. When the set of reference samples were tested using these primers, all were correctly identified to species based on the lowest Ct value. In addition, no instances of cross-species amplification were observed. The results of testing of reference tissue and faecal DNA samples are included in Appendix 3.

The sensitivity of the primers was tested by amplifying ten-fold dilution series of bat tissue DNA, from which standard curves were plotted (Fig.s 2.1 and 2.2). Standard curves were plotted for ten of the species in this study for which tissue DNA was available: *M. bechsteinii*, *M. daubentonii*, *M. mystacinus*, *M. nattereri*, *N. leisleri*, *P. nathusii*, *P. pipistrellus*, *P. pygmaeus*, *P. auritus*, and *R. hipposideros*. Standard curves could not be plotted for the remaining species as only faecal DNA was available, for which the target DNA cannot be accurately measured due to the presence of DNA from prey insects, gut bacteria, etc. The quantity of tissue DNA available for *Myotis myotis* was insufficient for the creation of a standard dilution series.

Using the standard curves, the R^2 value and the slope of the curve for each primer set were calculated using Microsoft Excel. All of the primer sets had R^2 values of 0.99-1.00.

Using the value of the slope of the standard curve, the percent efficiency of each primer set was calculated. Nine of the ten primer sets fell within the range of 90-110% efficiency, which is considered to be acceptable by Applied Biosystems (Table 2.3).

Table 2.2: Forward and reverse primer sequences for each species, with predicted amplicon length and melting temperature.

Species	Primer	Sequence	Amplicon
Barbastella barbastellus	BbarcytbF BbarcytbR	CACCTCCTATTCCTACACGAAACA GGGTGGAATGGGATTATATCTACG	Length: 80 bp T_M: 80°C
Eptesicus serotinus	EsercytbF EsercytbR	GGCTCTTTCTAGCCATGCACTAC TTACGTCTCGGCAGATGTGAGTA	Length: 78 bp T_M: 80°C
Myotis alcathoe	MalccytbF MalccytbR	GGCACAAGCCTTGTAGAATGA GAAGGCGAAAAATCGTGTTAGA	Length: 75 bp T_M: 78°C
Myotis bechsteinii	MbeccytbF MbeccytbR	ACAATCCAATAGGAATCCCCTCTA CTAATAGGCCGAGGATGTCTTTG	Length: 83 bp T_M: 77°C
Myotis brandtii	MbracytbF MbracytbR	CAATTCCGTACATTGGAACAGACCTT CGGGTCAAAGTAGCTTTGTCAACA	Length: 76 bp T_M: 78°C
Myotis daubentonii	MdaucytbF MdaucytbR	CTCTTATCTGCAATCCCATATATTGGC GGGTGGCCTTATCAACGGAA	Length: 79 bp T_M: 78°C
Myotis myotis	MmyocytbF MmyocytbR	CGAGACGTAAACTACGGCTGAGTA GAAGGTACAGGCAAATAAAGAATATTGAG	Length: 79 bp T_M: 78°C
Myotis mystacinus	MmyscytbF MmyscytbR	TTCCTAGCTATACACTATACGTCAGATACT GCGTAGGACTCAGCCGTAA	Length: 93 bp T_M: 78°C
Myotis nattereri	MnatcytbF2 MnatcytbR2	CGAGATGTAAACTATGGCTGAGTG TCCCCGTCCTACATGAAGATATAA	Length: 93 bp T_M: 74°C
Nyctalus leisleri	NleicytbF NleicytbR	TTGGAACAGATCTTGTTGAATGAATC GAAAGGCGAAAAATCGAGTTAGAGTA	Length: 78 bp T_M: 77°C
Nyctalus noctula	NnoccytbF NnoccytbR	GCCGACCTTGTTGAGTGAATTTGA AAGTGAAAGGCGAAAAATCGAGTTAGG	Length: 77 bp T_M: 79°C
Pipistrellus nathusii	PnatcytbF PnatcytbR	CAATTTACTCTCCGCAATCCCA GGTGGCTTTATCTACAGAAAAACCA	Length: 82 bp T_M: 78°C
Pipistrellus pipistrellus	PpipcytbF PpipcytbR	AACCGCCTTCAGCTCCGTTACT CGTGTAGGTATCGTAGAACTCATCCG	Length: 71 bp T_M:79°C
Pipistrellus pygmaeus	PpygcytbF PpygcytbR	GGATCCCTATTAGGCATCTGTCTAGGGCTG CTGAAGGCTGTTGCTGTATCTGACGTGTAG TGTATA	Length: 92 bp T_M: 77°C
Plecotus auritus	PaurcytbF PaurcytbR	TGCAATCCCATATATTGGAACAAGC AGTTAGTGTTGCTTTATCTACGGAGAAG	Length: 76 bp T_M: 77°C
Plecotus austriacus	PauscytbF PauscytbR	CGTATATTGGAACAACTCTAGTAGAATGA GGAATGCGAAGAATCGAGTC	Length: 79 bp T_M: 79°C
Rhinolophus ferrumequinum	RfercytbF RfercytbR	GAGCAACAGTTATCACAAACCTTCTC CGCCTCAGACTCATTCGACT	Length: 75 bp T_M: 79°C
Rhinolophus hipposideros	RhipcytbF RhipcytbR	TGCCTGGCCATACAAATCCTT GCTGTGTCGGTGTCTGATGTG	Length: 68 bp T_M: 79°C

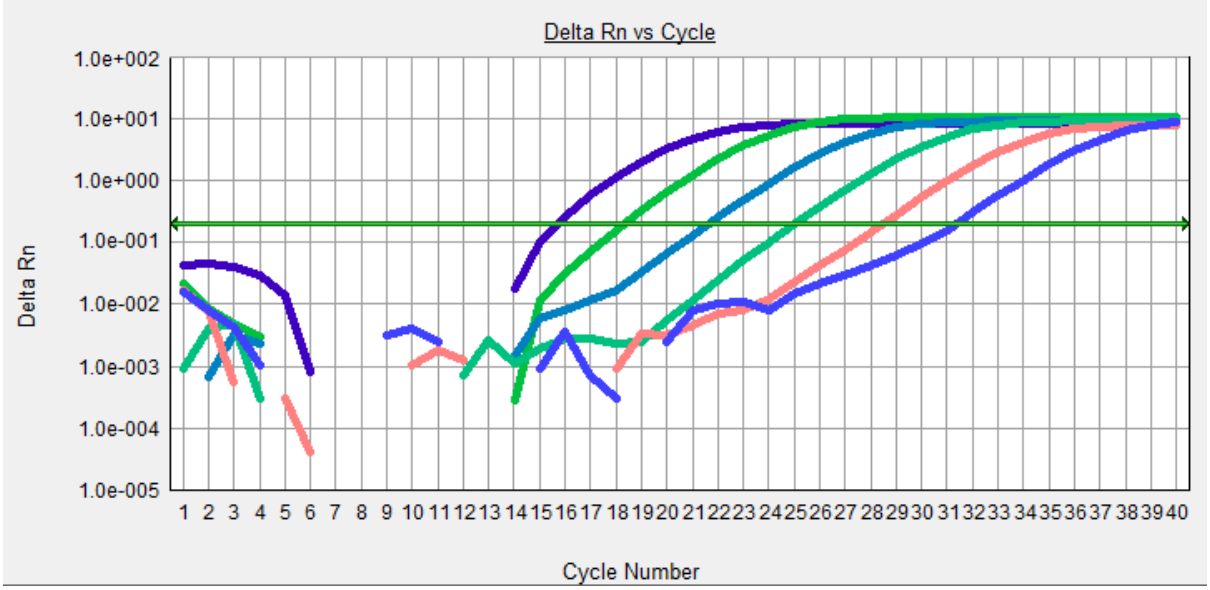

Figure 2.1: Example graph of amplification of dilution series for *P. pipistrellus,* starting at 4ng/µl (dark blue, at left), with subsequent dilutions progressing to the right.

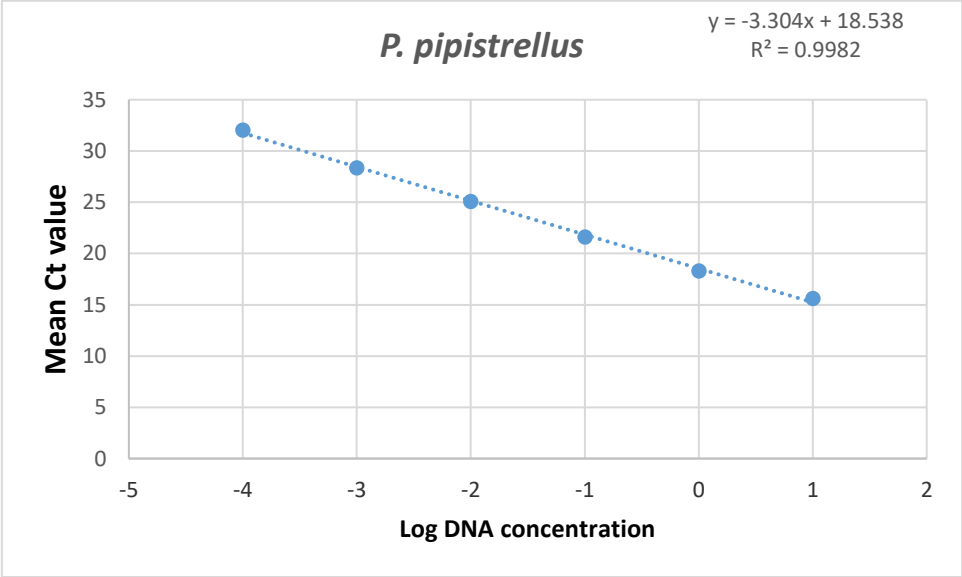

Figure 2.2: Example standard curve plotted for *Pipistrellus pipistrellus*, showing mean Ct value plotted against log DNA concentration.

Table 2.3: Table showing standard curve gradient, percent efficiency and R^2 value for each primer set analysed.

Species	Slope	% efficiency	R^2
M. bechsteinii	-3.2664	102.37	0.99
M. daubentonii	-3.289	101.39	1.00
M. mystacinus	-3.3659	98.2	0.99
M. nattereri	-3.2957	101.11	1.00
N. leisleri	-3.4467	95.04	0.99
P. nathusii	-4.128	74.68	0.99
P. pipistrellus	-3.304	100.75	1.00
P. pygmaeus	-3.2167	104.59	0.99
P. auritus	-3.3477	98.94	1.00
R. hipposideros	-3.2997	100.94	1.00

2.3.2 Roost site surveys

In total, 121 potential bat roost sites were surveyed in County Waterford. Of these, either definite bat signs (i.e. sightings of live bats or bat carcasses) or potential bat signs (i.e. probable bat droppings) were found at 73 of these sites, and DNA samples (either bat carcasses or droppings) were collected from 62 of these. Figure 2.3 shows the distribution of the sites where signs of bats were found, and Table 2.4 gives a breakdown of the types and number of sites where bat signs were found. Of the 73 probable bat roosts identified, only eight were already known to contain bats, with the other 65 roosts being previously unrecorded.

In addition to the main County Waterford survey, samples were obtained from 33 roosts in Counties Kildare, Galway and Wexford, giving a total of 95 sites from which bat DNA samples were obtained. A breakdown of the roosts of each type is given in Table 2.4.

Figure 2.3: Map of study areas. Left: sites in County Waterford where signs of bats were found (n = 73). Right: Map of Ireland showing counties from which bat DNA samples were obtained (GY- Galway, KE- Kildare, WD-Waterford, WX- Wexford).

Table 2.4: Breakdown of number of each type of potential roost site surveyed in Co. Waterford, number of these where definite or probable signs of bat presence were found (e.g. sighting of bats, bat droppings), and number of sites from which DNA samples could be collected (including samples from other counties).

Site type	No. surveyed	No. containing probable bat signs	No. sites DNA samples collected (Waterford)	No. sites DNA samples collected (other counties)
Church	78	45	42	13
Underground sites	9	6	6	0
Bat box	18	7	4	14
Other sites	16	15	10	6
Total	**121**	**73**	**62**	**33**

In total, 169 DNA samples were obtained from 95 sites (Table 2.5). At some sites, several samples were collected from different parts of the structure to increase the chance of detecting cases where multiple species were present in the same site. The majority of sites were visited on several occasions in successive years to collect samples, in order to increase the chance of detecting multiple species and to confirm whether sites were being used consistently by bats from year to year.

Table 2.5: Breakdown of number of DNA samples collected according to site type.

Site type	No. sites sampled	No. samples collected
Church	55	92
Underground	6	23
Bat box	18	24
Other	16	30
Total	**95**	**169**

When the DNA samples collected were tested using the real-time PCR primers for Irish bat species, 124 samples, or 73% (out of a total of 169), were identified as having originated from bat species. Only 17% of samples from underground sites were identified to species, compared to 79-92% of samples identified to species from the other site types (Table 2.6).

Due to the collection of multiple samples from each site, it was possible to identify bat species present at 89% of roosts. Bat species present at underground roosts were identified at 50% of sites, whereas bat species were identified at 91-94% of other roost categories. Results of real-time PCR testing of field samples are shown in Appendix 4.

Forty-five samples which could not be identified to species using the bat real-time PCR primers were tested for non-target small mammal species. Of these, only one yielded a positive result for wood mouse, *Apodemus sylvaticus*.

Table 2.6: Species identification success by sample and by site using the bat real-time PCR primers designed in this study, broken down by site type.

Site type	No. sites	No. sites species IDed (%)	No. samples	No. samples species IDed (%)
Church	55	50 (91%)	92	73 (79%)
Underground	6	3 (50%)	23	4 (17%)
Bat box	18	17 (94%)	24	22 (92%)
Other	16	15 (94%)	30	25 (83%)
Total	**95**	**85 (89%)**	**169**	**124 (73%)**

Each occurrence of a bat species at a particular site was classed at a separate roost. In total, 106 roosts were identified at the 84 sites where the bat species present were identified, based on analysis of faecal DNA samples collected. The majority of sites contained a single species, at 76% (n = 65). 20% of sites (n = 17) were found to be inhabited by two bat species, and 4% (n = 3) contained three species.

Seven bat species were identified in the roosts surveyed: *Pipistrellus pipistrellus, P. pygmaeus, Plecotus auritus, Myotis daubentonii, M. nattereri, M. mystacinus* and *Nyctalus leisleri*. *P. pipistrellus, P. pygmaeus* and *P. auritus* were the three most commonly encountered species overall, making up 77% of the total number of roosts recorded. The overall species composition of all of the roosts surveyed via DNA analysis is shown in Figure 2.4.

Distinct patterns of species occupancy were apparent in the different roost types (Figure 2.5). Church roosts were dominated by *P. pygmaeus, P. pipistrellus,* and *P. auritus,* although all seven bat species were detected. Underground sites contained exclusively *M. nattereri* and *P. auritus*. Bat boxes were dominated by *P. pygmaeus* and *N. leisleri,* although *P. pipistrellus* and *P. auritus* were also found. Six species (*P. pipistrellus, P. pygmaeus, P. auritus, M. nattereri, M. mystacinus* and *M. daubentonii*) were found in the other roost sites, with no species being predominant.

In addition to the bat roosts identified using non-invasive genetic sampling, 17 other bat roosts were discovered in Co. Waterford by means of daytime sightings or bat detector surveys. The species discovered at these roosts included *P. pipistrellus* (n = 5), *P. pygmaeus* (n = 4), *Pipistrellus* sp. (n = 1), *P. auritus* (n = 1), *M. nattereri* (n = 1), *M. daubentonii* (n = 1), *Myotis* sp. (n = 1) and *N. leisleri* (n = 3). The locations of these roosts are listed in Appendix 5.

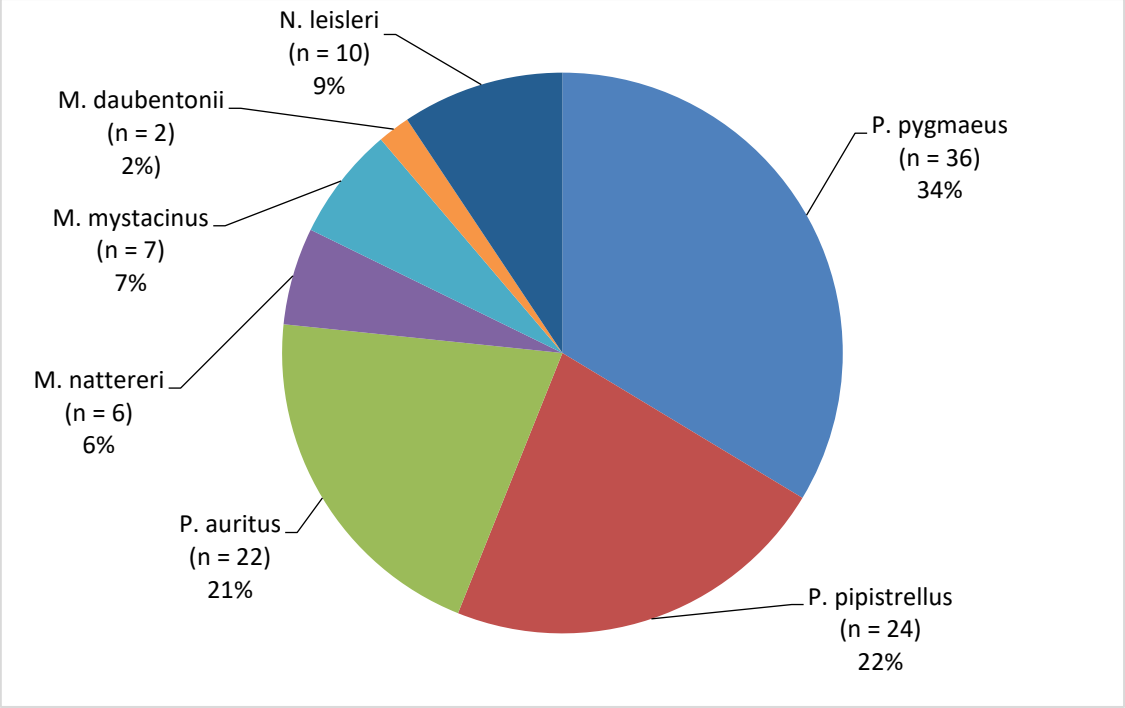

Figure 2.4: Bat species identified in all roosts based on analysis of faecal DNA samples.

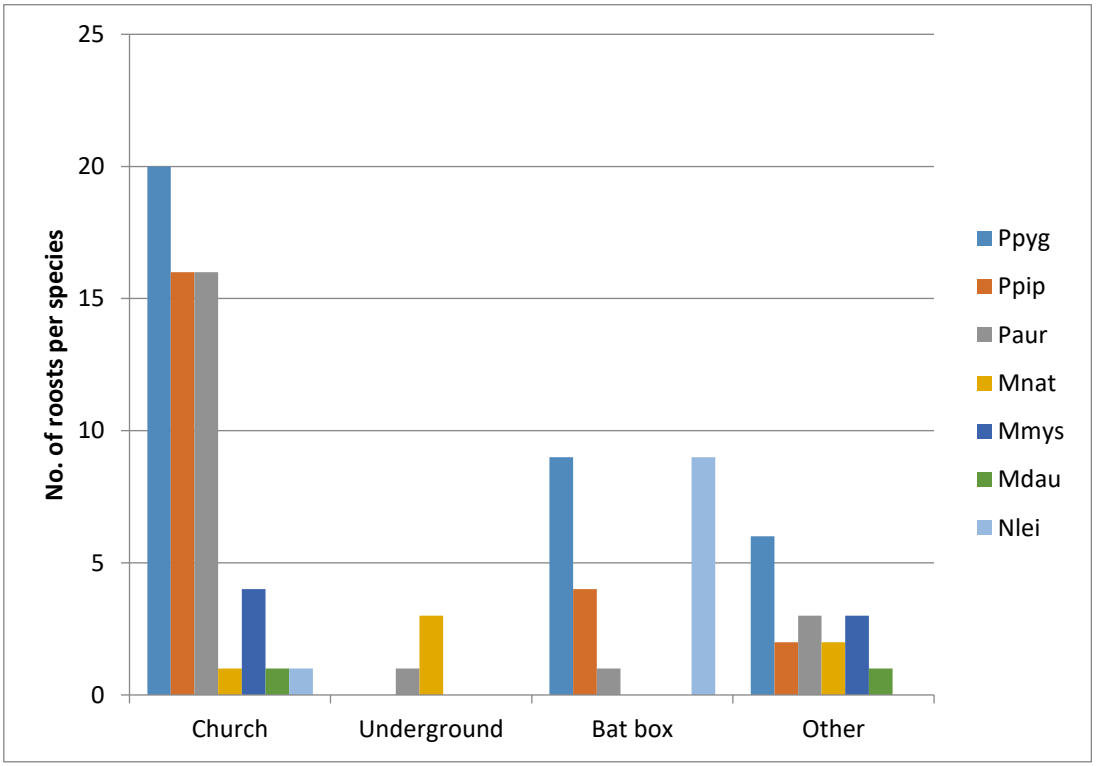

Figure 2.5: Species composition of different roost types surveyed, based on DNA analysis of dropping samples.

2.4 Discussion

The real-time PCR assays developed in this study successfully identified the species of origin of the 81 reference samples used, and all were found to be species-specific. These assays were also successfully used in a non-invasive survey of bat roosts across Ireland using faecal DNA samples. These assays have the potential to be used for similar surveys in the British Isles and across Europe.

A large number of new roost records were obtained for seven Irish bat species across four counties, providing additional distribution data for these species. The bat species encountered varied between the three main roost types surveyed (churches, underground sites and bat boxes), with the patterns seen in this study matching the known preferences of these species. Church roosts were mainly occupied by *Pipistrellus pipistrellus*, *P. pygmaeus* and *Plecotus auritus*, which were also found to be the main species present in other surveys of church roosts in England and Ireland (Sargent, 1995; Roche, 1998). However, while *M. nattereri* is also known to be a common species occurring in churches in England (Zeale et al., 2016), only a single church roost of this species was found in this study, and no *M. nattereri* roosts were discovered in Roche's (1998) survey of churches in Ireland either. A possible reason for this may include some aspect of church architecture in England making them more favourable for *M. nattereri* than in Ireland. This may be potentially related to their age, as Zeale et al.'s (2016) study of *M. nattereri* in English churches noted that all were originally built in the Medieval period (i.e. pre-1500), in comparison to the Irish churches in this study which for the most part were built between the mid-1700s and 1900 (National Inventory of Architectural Heritage, 2018). It is also possible that *M. nattereri* is simply more common in England, with an estimated population of 321,000 (Mathews et al., 2018), but as there is no population estimate for Ireland due to a lack of data (Roche et al., 2014) a direct comparison cannot be made.

The only species encountered in underground sites were *P. auritus* and *Myotis nattereri*, both of which are known to commonly use such sites as hibernation roosts and swarming sites, as well as other *Myotis* species (Dietz et al., 2009). *Pipistrellus pipistrellus*, *P. pygmaeus* and *Nyctalus leisleri* were predominant in the bat box roosts. The same pattern was observed by Poulton (2006) in an analysis of a large dataset of records of bat box inspections from across the British Isles, with 74% of bat records accounted for by these three species.

Seven *M. mystacinus* roosts (all in Waterford) and six *M. nattereri* roosts (one in Kildare and the remainder in Waterford) were discovered during this survey, none of which had previously been recorded. This is a substantial increase in the number of known locations for these species in Waterford, as only two roosts for both species were previously known (Roche et al., 2014). This is also significant nationally, as only 41 *M. mystacinus* roosts and 66 *M. nattereri* roosts have been previously recorded in Ireland as a whole (Roche et al., 2014). As these species are thought to be under-recorded in Ireland, non-invasive genetic surveys of potential roost sites in areas of favourable habitat for these species could provide more data on their distribution in Ireland.

While the set of real-time PCR assays identified the bat species of origin of the majority of the samples collected in the field, 26.6% of the putative bat dropping samples (n = 45) could not be identified to species. Perhaps the most likely reason for PCR failures was DNA degradation in samples due to environmental conditions or the age of the samples collected, which is one of the main drawbacks of using non-invasive samples for genetic studies, including bat droppings (Taberlet et al., 1999; Puechmaille et al., 2007). High humidity is known to be a major factor causing DNA degradation in bat dropping samples, whereas dessicated bat droppings can maintain good DNA quality for several years (Puechmaille et al., 2007; Boston et al., 2012). The cool, humid environment encountered in the underground sites surveyed in this study is the most probable explanation for the poor PCR amplification seen in samples from these sites (17%) as the DNA present in many of these samples had likely become degraded, and samples from underground sites represented 42% of all samples where PCR amplification failed. In contrast, samples collected from bat boxes showed the highest rate of PCR amplification, probably because the bat droppings deposited in bat boxes were subjected to warm, dry conditions which helped to preserve the DNA contained within them.

A second possible reason for the failure of PCR amplification using the bat species identification assays may be that samples were collected from non-target species. However, when these were tested using real-time PCR assays for the identification of small mammal species, which were thought to be most likely non-target species to be encountered, only a single sample was found to originate from a small mammal (specifically a wood mouse, *Apodemus sylvaticus*). This indicates that non-target small mammal species contributed very little to the non-identification of samples with the bat species assays. Another possibility is that some unidentified samples may have originated from non-target bird species, in particular swallows (*Hirundo rustica*) and house martin (*Delichon urbicum*), which were seen nesting at

several sites. While it was thought unlikely that droppings from these species could have been confused for bat droppings during field surveys, they do show some similarity to bat droppings due to their high content of insect remains, so it is possible that some of the unidentified samples may have originated from these bird species. However, as PCR primers for the identification of swallows and house martins were unavailable, it was not possible to test unidentified samples for these species.

The high sensitivity of real-time PCR assays to the small quantities of DNA makes them especially well-suited to identifying samples from non-invasively collected samples. In addition, as well as confirming the species of origin, the Ct values obtained can provide an assessment of the relative quantity of target DNA in a sample. This approach was used by O'Neill et al. (2013) in a non-invasive study of otters (*Lutra lutra*), where the highest quality samples as measured by the Ct values of a real-time PCR species identification assay were selected for sex typing and genotyping analysis.

It should be acknowledged that the set of real-time PCR species identification assays designed in this study may have some limitations on their use in certain situations. Firstly, samples obtained from species not resident in Great Britain and Ireland would not be identifiable using this set of assays. This may arise in the case of vagrant bat species which have been known to occasionally arrive in Great Britain from mainland Europe, such as Kuhl's pipistrelle, which has been recorded in Great Britain ten times since 1991 (Bat Conservation Trust, 2010). In this case DNA sequencing would be required to identify the species of origin, but this is likely to be a relatively rare occurrence. Secondly, while this set of assays has the potential to be used across Europe, some redesign of primer sets may be required to ensure specificity to the target species. As these assays were only designed within the context of the set of the 18 resident British bat species and did not consider other bat species, it is possible some of the primer sets may show cross-species amplification with other European bat species not native to Great Britain. Thirdly, the phylogeny of European bats as a whole is incompletely understood, with new cryptic bat species still being discovered. There are also instances where hybridisation between species is known to have occurred, for example between the greater mouse-eared bat, *Myotis myotis*, and lesser mouse-eared bat, *Myotis blythii*, such that some populations of these morphologically distinct species share the same mtDNA haplotypes (Berthier et al., 2006). While there are no known cases of further cryptic species or hybridisation between bat species in Great Britain and Ireland, these may pose a problem for the use of the species identification assays designed in this study. As these assays are designed to target the mtDNA Cyt b gene,

any given assay would be unable to distinguish between different species which share mtDNA haplotypes.

Although there are some limitations associated with this set of assays, most of the drawbacks outlined above do not apply within the British Isles. It should also be noted that these limitations apply equally to the assays published by Boston et al. (2011) and Hamilton et al. (2015). Therefore, the set of bat species identification assays designed in this study will provide a useful novel tool for bat surveyors in Ireland and the UK, and (in conjunction with other methods such as DNA sequencing) could provide the basis for a comprehensive set of assays for the entire bat fauna of Europe.

2.5 Conclusions

- Real-time PCR species identification assays were successfully designed and validated for the seventeen resident bat species of the British Isles, as well as one recently extinct bat species.
- These real-time PCR assays were successfully used in a large-scale field survey of bat roosts, using non-invasive samples.
- A large number of previously unrecorded bat roosts were discovered, greatly improving the known distribution of several bat species in Waterford, especially for two rare bat species, *Myotis nattereri* and *M. mystacinus*.

Chapter 3

Design of real-time PCR sex typing assays for the lesser horseshoe bat, *Rhinolophus hipposideros*

3.1 Introduction

The ability to sex type non-invasive DNA samples from wild animals is a crucial tool for researchers, which can reveal much information about a species' social structure, population size and dynamics. While species-specific sex typing assays have been designed and applied to field studies of many wild mammal species, bat species have been relatively overlooked in this regard.

Studies which have examined sex typing assays for bat species have mainly relied on using several previously designed primers which target conserved regions of sex chromosome genes, which have been applied to a wide range of mammal species. These studies have tested several sex chromosome genes for their utility in sex typing of bats, including ZFX, ZFY, SRY, DBY and SMCY (Bryja and Konecny, 2003; Bullejos et al., 2000; Hellborg and Ellegren, 2003; Korstian et al., 2013).

In relation to *Rhinolophus hipposideros* specifically, to date three studies have applied sex typing assays to the sex identification of individuals of this species. Dool (2010) used primers designed by Hellborg and Ellegren (2003) to target the DBY3 region to identify male bats. This was carried out to determine the sex of certain tissue samples of uncertain sex, which were being used as part of a study of the phylogeography and population genetics of the lesser horseshoe bat in Europe.

Afonso et al. (2016) used two different primer sets to sex type lesser horseshoe bats. The authors used primers designed by Aasen and Medrano (1990) to target the ZFX and ZFY genes as a positive control, and primers designed by Sanchez et al. (1996) targeting the SRY gene in order to identify male animals. This study reported being able to successfully sex type 145 out of 232 genetically identified lesser horseshoe bats, from faecal pellets collected at a number of roosts.

The most recent study was carried out by Zarzoso-Lacoste et al. (2018), who examined the sex ratio of lesser horseshoe bat colonies in nineteen roosts in northern France. Zarzoso-Lacoste et al. (2018) developed a single PCR primer set designed to simultaneously target sections of the 8^{th} intron of both the DBX and DBY genes, producing a single band in females and two bands in males when visualised via gel electrophoresis.

None of the studies mentioned have used real-time PCR technology, which has the advantage of being sensitive to small quantities of target DNA and thus is very useful for application to non-invasive genetic studies. The aim of this study was to design real-time PCR primers for the sex typing of lesser horseshoe bats, building on the broadly applicable PCR primers described above, focusing on the ZFX, ZFY, SRY and DBY genes.

3.2 Materials and Methods

3.2.1 Sample collection and DNA extraction

Tissue samples were obtained from eight lesser horseshoe bat specimens (Table 3.1). A small section (approx. 5 mm diameter) of wing tissue was cut off from each bat being sampled with a scissors and tweezers, which were flamed between samples to prevent cross-contamination. The tissue DNA was then extracted using the ZR Genomic DNA™ Tissue MicroPrep (Zymo Research, cat. no. D3051) according to the Solid Tissue protocol, with Zymo-Spin™ II columns (Zymo research, cat. no. 3041). DNA extracts were stored at -20°C. As all of the tissue samples were obtained from dead bats, their sex was unknown prior to DNA testing.

Table 3.1: List of *R. hipposideros* specimens or tissue samples obtained for sex chromosome gene analysis.

***R. hipposideros* individual**	**Place of origin**	**Collected/ donated by**
A	Unknown location, Ireland	A. Collins
B	Pencelli Mill, Powys, Wales	D. Jermyn
C	Plas Llywngwern, Powys, Wales	D. Jermyn
D	Buckland, Powys, Wales	D. Jermyn
E	Llangovan, Monmouthshire, Wales	D. Jermyn
F	Ffrwdgrech, Powys, Wales	D. Jermyn
G	William King House, Kilgarvan, Co. Kerry, Ireland	A Harrington
H	Plas Llywngwern, Powys, Wales	D. Jermyn

In addition to tissue samples, faecal DNA was extracted from bat droppings collected at seven known *R. hipposideros* roosts in Counties Mayo (n = 1), Galway (n = 2), Clare (n = 1) and Kerry (n = 3). DNA was extracted as described in section 2.2.1.2, from 94 individual faecal pellets.

All tissue and faecal DNA samples were subjected to a real-time PCR species identification assay, RhipCytbF/R, as described in Chapter 2. Tissue DNA samples were diluted to a standard concentration of 4ng/µl prior to testing. Testing with the species identification assay was carried out in order to ensure that samples originated from *R. hipposideros* in the case of the faecal DNA samples. In addition, the Ct values obtained from this assay provided a relative measure of the quantity of *R. hipposideros* DNA present in each sample, allowing the faecal DNA samples containing the highest amount of target DNA to be selected for further use.

3.2.2 PCR

As no published sequences of *R. hipposideros* sex chromosome genes could be found on the GenBank (NCBI) database (Clark et al., 2016), the primer sets described in Table 3.2 were used to generate PCR products in order to obtain sequence data of the ZFX, ZFY, SRY and DBY genes.

PCR reactions were carried out with a reaction mix of 5 µl of GoTaq® Hot Start Green Master Mix (Promega), 5 µM of each primer, 2 µl of DNA and 2 µl of water to a total volume of 10 µl. Negative controls contained water instead of DNA. PCR reactions were carried out using an Applied Biosystems 2720 thermal cycler. The PCR protocol used was a touchdown-based programme as per Hellborg and Ellegren (2003) for the LGL331/335, RhipLGL-Y, SRY-hmg, DBY3, DBY7, and DBY8 primer sets. Additional PCR reactions using different protocols were used for the certain primer sets. For the SRY-HMG primer set a PCR protocol described by Afonso et al. (2016) was carried out. For the DBY7 and DBY8 primers, additional reactions were carried out using the TDOWNHI protocol used by Dool (2010) for sex typing of *R. hipposideros,* with the following conditions: 95°C for 10 minutes, followed by 10 cycles of 95°C for 15 s, 65°C for 30 s and 72°C for one minute, with the annealing temperature decreasing by 2°C every two cycles, with a final extension at 72°C for five minutes.

PCR products were separated and visualised on a 2% agarose gel stained with ethidium bromide (0.25 µg/ml), with 4 µl of each PCR product being used for visualisation. The gel was prepared by dissolving 1.5 g of agarose (Sigma, cat. no. A9539) in 75 ml 1 X TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) at approximately 60°C, and 1µl of ethidium bromide (Sigma, cat. no. E1510) was added for fragment visualisation under UV light using GeneSnap V6.10 image analysis system (SynGene).

Table 3.2: List of previously published primers used in this study to obtain sex chromosome gene sequences for *R. hipposideros*.

Gene	Primers	Primer sequence (5'-3')	Target species	Authors
ZFX/ ZFY	LGL-331/ LGL-335	F: CAAATCATGCAAGGATAGAC R: AGACCTGATTCCAGACAGTACCA	*Odocoileus spp.*	Cathey et al. (1998)
SRY	SRYhmg-F/R	F: GTCAAGCGCCCCATGAATGCAT R: AGTTTGGGTATTTCTCTCTGTG	Insectivore spp.	Sanchez et al. (1996)
DBY3	DBY3F/R	F: ACTATCGACAGAGYAGTGGTT R: TCCATAACCATCAYTATTGTAG	Mammal spp.	Hellborg and Ellegren (2003)
DBY7	DBY7F/R	F: GGTCCAGGAGARGCTTTGAA R: CAGCCAATTCTCTTGTTGGG	Mammal spp.	Hellborg and Ellegren (2003)
DBY8	DBY8F/R	F: CCCCAACAAGAGAATTGGCT R: CAGCACCACCATAKACTACA	Mammal spp.	Hellborg and Ellegren (2003)

3.2.3 Sequencing and Bioinformatics

PCR products deemed to be suitable for sequencing were purified using microClean® (CamBio, cat. no. 2MCL-10), according to the manufacturer's instructions. The DNA sequencing of the PCR products was outsourced to Source Bioscience Ltd. The protocol followed Sanger sequencing based on BigDye chemistry (Applied Biosystems). The nucleotide sequences obtained were analysed using Sequence Scanner Software 2.0 (Applied Biosystems) and were compared with published sequences available in the GenBank (NCBI) database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). Where several sequences were obtained, these were aligned in MEGA 6.0 using the Clustal W algorithm in order to compare sequences and identify possible base call errors.

DNA sequence data, from both of nuclear and mitochondrial DNA genes, can provide valuable information on the evolutionary history and taxonomic relationships of species and higher-order taxa, or phylogenetics (Brito and Edwards, 2009). Phylogenetic studies typically use sequence data from several genes simultaneously to create a consensus phylogenetic tree as inferred phylogenetic histories may vary between markers, either due to differing mutation rates and modes of inheritance (such as the difference between mitochondrial and nuclear DNA), and because episodes of hybridisation between species can result in different markers showing varying phylogenetic histories, known as gene tree discordance (Degnan and Rosenberg, 2009). Thus, while many studies focus on sequences from several commonly-used genes for phylogenetic analysis, such as the Cyt b, ND1, 16S and RAG2 genes often used in

phylogenetic studies of bats (Mayer and Von Helversen, 2001; Ruedi and Mayer, 2001; Kiefer et al., 2002; Ibañez et al., 2006; Spitzenberger et al., 2006; Mayer, et al., 2007; Stadelmann et al., 2007; García-Mudarra et al., 2009), sequence data from other genes can also provide useful information and differing persepectives. For this reason, where possible the DNA sequence data obtained from *R. hipposideros* X chromosome and Y chromosome genes in this study was used to create phylogenetic trees to compare this species with the most similar published sequences from other closely related mammal species. Neighbour-joining trees were created in MEGA 6.0 using 1,000 bootstraps, and genetic distance was computed using the P-distance method (Nei and Kumar, 2000). For each tree, *R. hipposideros* sequence data was compared with sequences from the most closely related species identified by BLAST analysis, with a sequence from a more distantly related species used as an outgroup to root the tree.

3.2.4 Primer Design

Based on DNA sequence data obtained for *R. hipposideros* sex chromosome genes, several real-time PCR primer sets were designed to target sequences specific to both the X chromosome (to act as an internal positive control) and the Y chromosome. Real-time PCR primers were designed using Primer Express v2.0 (Applied Biosystems), based on SYBR Green I chemistry and targeted species-specific nucleotide polymorphisms at the 3' ends of both primers. The design parameters used included a melting temperature of between 58°C and 60°C, primer length of between 20 and 30 bp, a GC content of between 45% and 55% and the production of an amplicon between 70 and 100 bp long.

Primer sets were checked using BLAST searches to ensure that they were specific to the target species. Primers were ordered from Eurofins (Germany) in a lyophilised state and were resuspended in sterile water to a stock concentration of 100 pmol/µl. Aliquots were diluted with H_2O to a working concentration of 5 pmol/µl (5 µM) each of both forward and reverse primers for use in the real-time PCR reactions.

3.2.5 Real-time PCR

Once real-time primers were designed, these were tested using both tissue and faecal DNA samples to investigate their effectiveness in differentiating between samples originating from male and female animals. Real-time PCR reactions using SYBR Green I-based assays were carried out as per section 2.2.1.4. However, the number of cycles was increased from 40 to 50 to reflect the lower quantity of nDNA which was being targeted, compared to mtDNA.

3.3 Results

3.3.1 ZFX/ZFY genes (LGL-331 and LGL-335 primers)

This primer set was designed by Cathey et al. (1998) to produce an 800-900bp PCR product in North American deer species (*Odocoileus* spp.), with exact product sizes varying between species. Male individuals were expected to display two bands of slightly different sizes. All six *R. hipposideros* samples tested using this primer set (individuals A-F) were successfully amplified (Fig. 3.1). However, the two bands which were expected to be seen in male individuals could not be discerned, with all individuals displaying a single broad band of about 800bp. Although only a single band was seen, it was thought that the broadness of these bands could represent two different PCR products very similar in size, so sequences were obtained from individuals A, B and C, ranging from 797- 801bp long.

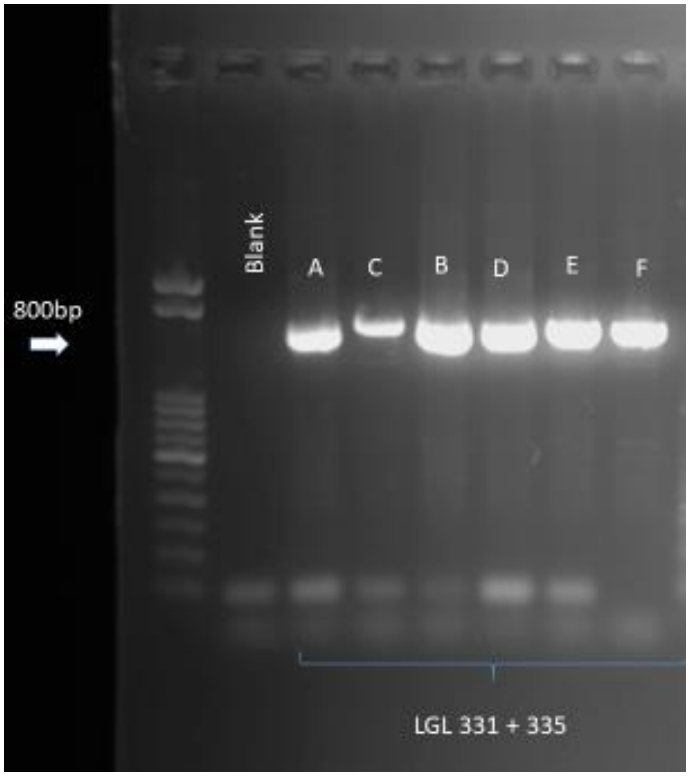

Figure 3.1: Amplification of *R. hipposideros* samples using the LGL331/335 primer set. *R. hipposideros* individuals labelled A-F. 4 µl of each product was inserted per well, on a 2% agarose gel.

One high quality sequence of 798 bp was obtained from individual C (Fig. 3.2). This was found to be very similar to published ZFX sequences through BLAST analysis, with the closest

similarity being to the black rhinoceros (*Diceros bicornis*), at 87% identity (Table 3.3). A neighbour joining tree was constructed using this sequence and the most similar published sequences (Fig. 3.3).

GCTAAGAAGGAAAAGAAAGGTGCATGAGTGATCAAACCACGTTCTGTTCTGGTATCTTCA 60
AGAATTTAAAGTGTGTTCTGAACACTATTGGACAAGCACTGCTAAGTTAGGTTATTTGCT 120
ACTTAACATTCCTATTACCATTTTTCAACATAAGAGATATAGCAACCTCATCATAAAGAG 180
GAACCTGGTCTGGAAGCTTCATTCAGTAGGACTGTATCAATCTCACTCCCTCCGGTTTAA 240
AAAAAGAAATGAATAAATATGTAACGTCTGTCACTAGTGACTGAAGCTGTATCAATTTGG 300
AAATTGGTGGGAAATTATGCATGAGAATCAAGTTTCATAGTCACAATTTCTGCTTTGGTT 360
ATTCCAGAAAACAATTTATGTCCATTTACAGTAAAGCTTGAATATACCTATAAAATTTTT 420
AATATGTAACTCGGCATATGAGAATAGATAGGAATATGTCACATGAACTGAAGTCCCTGT 480
CCTGCTGCTCTGTAAATTATGCCTGCTTTATGCTGACATAATCAGATTCTTCAAATCTAT 540
TTACTTGGGGAAAAAAATCATTTTCAATTTTTTCATCCATGTTTGATCCATTCCATTTTA 600
TCCAAGGAAATCATTCATGAATATCACTGAATTCTTAAAATTATATTTTCCAATTCAATA 660
CACAAAAGCTACATGTGGTCTAGCAGCTGAAATGCCATCACAACACCTCTGTGGATACAT 720
ACTAGAGCTTCATCTGAGAGCTCGCAAAGCACGCTGCGTTGTGGGACTCATGTGCCCTCA 780
CCTGTTTGGTACTGTCTG 798

Figure 3.2: ZFX intron sequence obtained for *R. hipposideros* using the LGL-331/335 primer set. The position of real-time PCR primers designed based on this sequence are highlighted in blue (RhipZFX-F/R2) and green (RhipZFX-F/R3).

Table 3.3: BLAST search results for *R. hipposideros* ZFX sequence derived from the LGL331/335 primers.

ZFX product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Diceros bicornis*	Black rhinoceros	ZFX	95%	87%
2	*Ceratotherium simum*	White rhinoceros	ZFX	95%	87%
3	*Bos Taurus*	Domestic cow	ZFX	92%	84%
4	*Bos mutus*	Wild yak	ZFX	92%	84%
5	*Tapirus indicus*	Malayan tapir	ZFX	95%	84%
6	*Lepilemur septentrionalis*	Northern sportive lemur	ZFX	68%	84%
7	*Lemur catta*	Ring-tailed lemur	ZFX	67%	84%
8	*Lepilemur dorsalis*	Grey-backed sportive lemur	ZFX	68%	83%
9	*Lepilemur ankaranensis*	Ankarana sportive lemur	ZFX	67%	83%
10	*Microcebus murinus*	Grey mouse lemur	ZFX	68%	83%

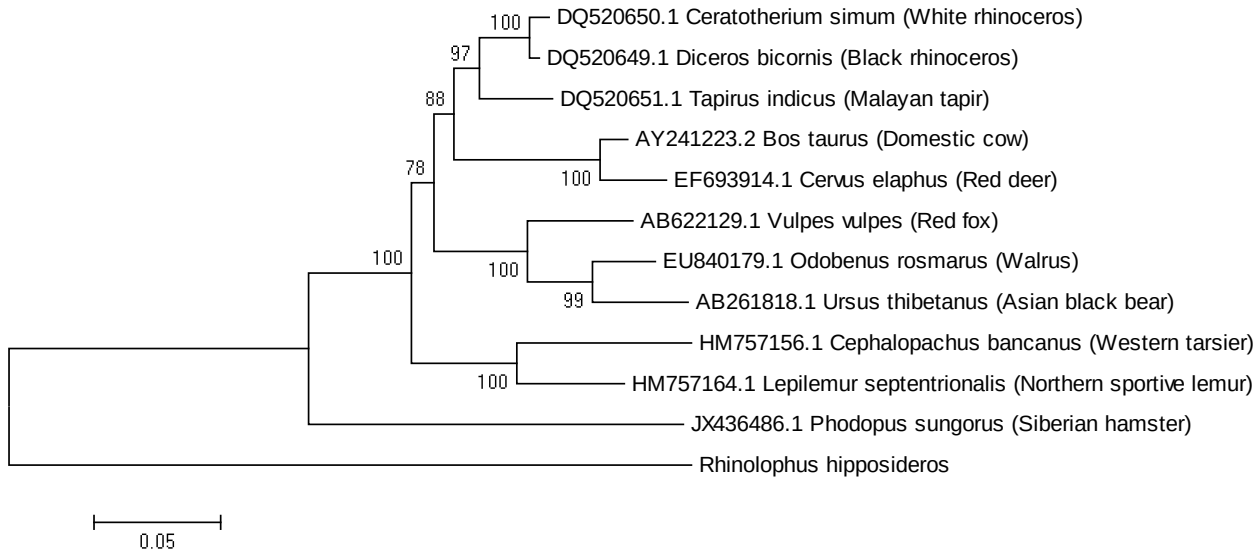

Figure 3.3: Neighbour joining trees of ZFX sequences of *R. hipposideros* and the most similar published sequences on GenBank. Neighbour joining tree were constructed using 1000 bootstraps and genetic distance was computed using the P-distance method (Nei and Kumar, 2000).

The other two sequences obtained were of poor quality, which possibly indicated that the ZFY sequence had also been amplified during PCR. A conventional PCR primer set (Rhip-LGLy-F/R) was designed to specifically target the potential *R. hipposideros* ZFY gene in order to obtain a good quality sequence (Fig. 3.5; Table 3.16). This primer set targeted either end of the ZFY sequence, which had already been partially obtained from the first set of PCR products. When six *R. hipposideros* tissue samples were amplified with this primer set, a single bright band about 700bp long was seen in individuals A, G and H, which were inferred to be the targeted males, with a number of faint bands seen in the other three individuals (Fig. 3.4). The products of these three samples were sequenced, resulting in high quality sequences which allowed a single 743bp sequence to be constructed by alignment in MEGA (Fig. 3.5).

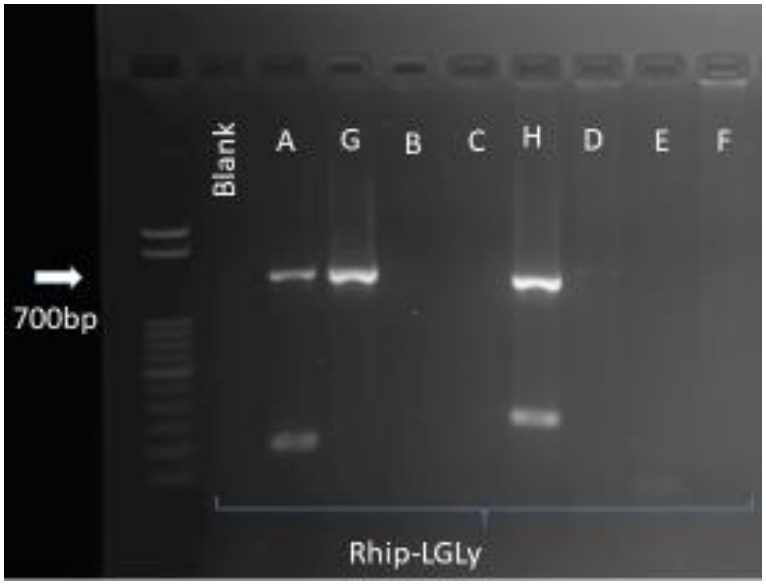

Figure 3.4: Gel image of products of PCR reaction of *R. hipposideros* individuals with Rhip-LGLy primer set. 4 µl of each product was inserted per well, on a 2% agarose gel.

TGCTAAGAAAGGAAAAGGAGTGTGAGTATACAAACAAATTCTATTTTTGATTTCAAGACA 60
AGTATTCCTAAATTAGGTTAATATTCCTTTTGTTCAACAAAAGATACAATAACCTAGTAA 120
TAAAGAGAATCTAGTCTAGAAATTTCATTATGACAGAACCAATCTCACAAAGTATCAATT 180
TAAAGTTACATAAATTTAGATATCTATTGAAAATCATTCATAAAAATAAAGTTCAACATT 240
ACAGCTTTGTTTATTCCAAGATGAATTTATGCTAATTTAAAGTTTAACCACACCTATAAA 300
GAAAACCTTAATATGCAAATCGTTTAACAAGGCAATACAGGAAAGGAATATGCCCAATGA 360
ACAACCACTCCTCTGTAATGTCTCACTCCTCTGTAAATGATTAAGGCTGACTGAAGTAGA 420
TTTTTTTTATATTTGTTCATTTAAAGTTAACTAACATATTTAGGGCTTTACTGTCATTT 480
CCAAAAAAATAACCTGAGATTTTAATTCTTTTTTCTGAATGTCTGATCCAAAAGCAATTT 540
CTTTTTATCTAAGAAAATCATTCATGAATATCAATGAATTCTTAAAATCATAATTTCAAA 600
TTATATGCACAAAAGCTACACATGGTCTAACAGCTAAAATGTTATCACAACACCTCTTTG 660
GAAACACACTAGAGTTTCATCTGAGAGCTCACAAAGCATGCTGTGCTATGGAACTCATGT 720
GCCCTCACCTGTTGGTACTGTCT 743

Figure 3.5: ZFY intron sequence obtained for *R. hipposideros* using the Rhip-LGLy-F/R primer set (highlighted in grey). The position of real-time PCR primers designed based on this sequence are highlighted in blue (RhipZFY-F/R2), green (RhipZFY-F/R3) and pink (RhipZFY-F/R/4).

BLAST analysis of this sequence showed that it was homologous to other published ZFY sequences, being most similar to a ZFY sequence from the tricoloured bat, *Pipistrellus subflavus* (Table 3.4). A neighbour joining tree was constructed using this sequence and the most similar published sequences (Fig. 3.6).

Table 3.4: BLAST search results for *R. hipposideros* ZFY sequences derived from the Rhip-LGLy primers.

ZFY product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Pipistrellus subflavus*	Tricoloured bat	ZFY	75%	79%
2	*Equus hemionus*	Onager	ZFY	90%	77%
3	*Equus przewalski*	Przewalski's horse	ZFY	90%	77%
4	*Diceros bicornis*	Black rhinoceros	ZFY	90%	76%
5	*Equus caballus*	Domestic horse	ZFY	58%	79%
6	*Saimiri boliviensis*	Black-capped squirrel monkey	ZFY	27%	83%
7	*Nomascus gabriellae*	Yellow-cheeked gibbon	ZFY	31%	77%
8	*Symphalangus syndactylus*	Siamang	ZFY	31%	77%
9	*Hylobates moloch*	Silvery gibbon	ZFY	31%	77%
10	*Hylobates agilis*	Agile gibbon	ZFY	31%	77%

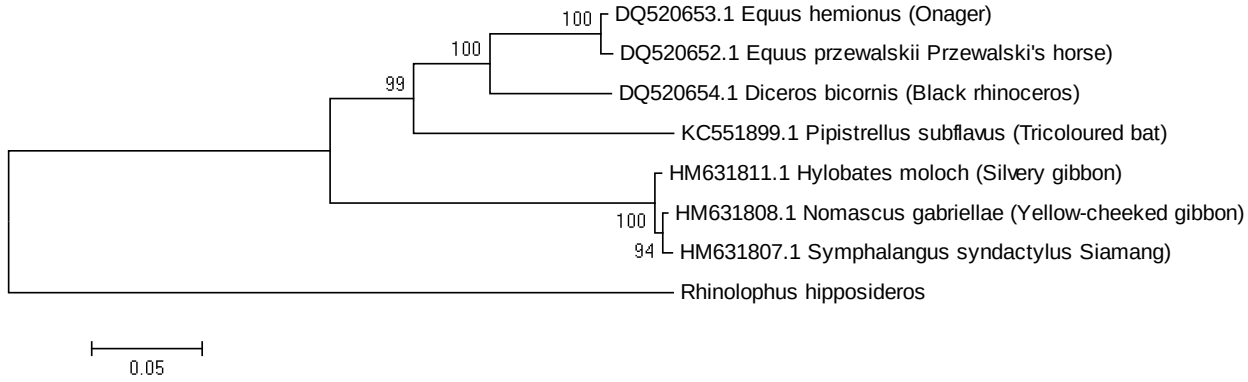

Figure 3.6: Neighbour joining trees of ZFY sequences of *R. hipposideros* and the most similar published sequences on GenBank. Neighbour joining tree were constructed using 1000 bootstraps and genetic distance was computed using the P-distance method (Nei and Kumar, 2000).

Using the sequence obtained from the LGL-331/335 primers, two sets of real-time PCR primers targeting the ZFX gene were designed, RhipZFX-F/R2 and RhipZFX-F/R3 (Fig. 3.2; Table 3.16). These were then tested using the eight tissue DNA samples available and ten faecal DNA samples. The first primer set appeared to show a distinct difference in the Ct values obtained from tissue and faecal samples, which would be expected due to the lower quantity of nDNA present in faecal samples. Some non-specific amplification occurred in the negative controls with this primer set. The RhipZFX-F/R3 primer set was designed in an effort to reduce this observed non-specific amplification. This appeared to be successful, as the observed Ct values for tissue and faecal samples were very similar to those seen in RhipZFX-F/R2, but the non-specific amplification seen in the negative controls had been decreased, as seen in a slight increase in the Ct value (Table 3.5). Ct values were also seen to increase as DNA template decreased using a dilution series (Fig. 3.7). Based on these values, the primer set RhipZFX-F/R3 was selected to act an internal control for further sex typing assays.

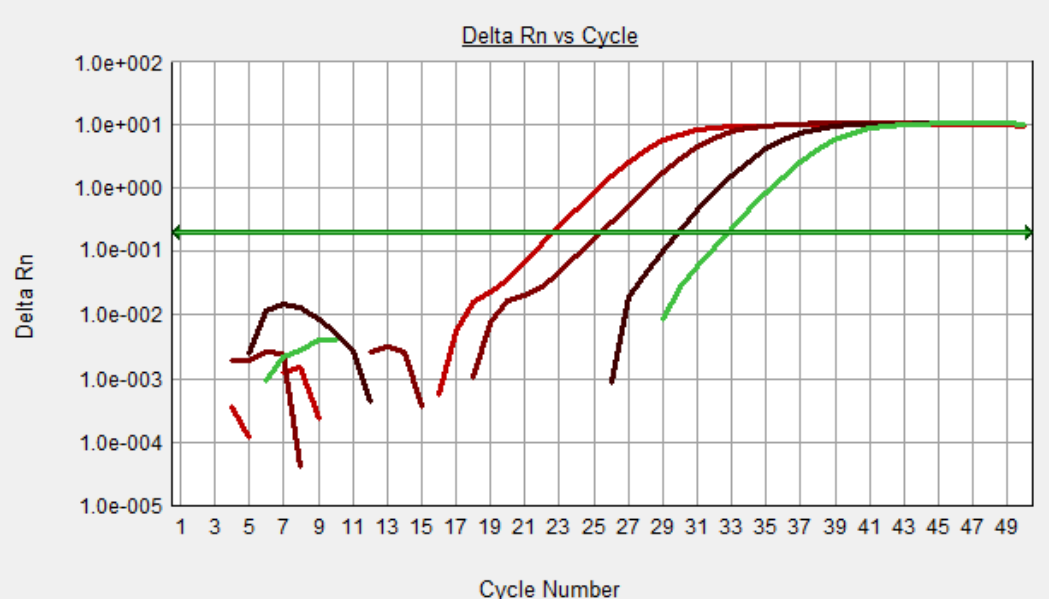

Figure 3.7: Real-time PCR amplification of a dilution series of a tissue sample using the RhipZFX3F/R primer set (red- 10 ng/µl, brown- 1ng/µl, black- 0.1 ng/µl, green- 0.01 ng/µl).

Three real-time PCR assays were designed to target the ZFY sequence obtained using the Rhip-LGLy primers, RhipZFY-F/R2-4 (Fig. 3.5; Table 3.16). None of these primer sets appeared to be effective in clearly identifying male individuals, with little difference being seen in Ct values between individuals or between tissue and faecal DNA samples (Table 3.5).

Table 3.5: Results of real-time PCR sex typing assays designed for *R. hipposideros*, based on sequence data obtained using the LGL331/335 and RhipLGLy primer sets. Negative amplification is indicated by "U" (for "undetermined").

Individual/faecal sample	Type	RhipCytb Ct	RhipZFX2		RhipZFX3		RhipZFY2		RhipZFY3		RhipZFY4	
			Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm
A	T	15.82	25.47	76.6	25	70.8	24.22	74.8	25.44	70.8	24.21	83.6
B	T	14.92	23.74	76.6	24.4	70.8	23.05	74.8	22.29	71.1	25.28	86.9
C	T	15.27	25.3	76.9	25.74	70.8	26.27	75.1	25.25	71.1	33.61	84.3
D	T	14.42	24.27	77.2	23.99	70.8	23.66	75.4	22.88	71.4	24.55	80.7
E	T	14.33	23.06	76.9	33.12	70.8	28.28	75.4	35.01	70.8	28.35	70.9
F	T	14.23	23.67	76.9	22.32	70.8	28.34	75.1	42.06	76.9	31.46	85.6
G	T	14.88	29.89	75.8	25.1	70.6	23.35	74.9	25.7	71.3	32.07	76.8
H	T	15.7	24.76	76.5	24.77	70.6	25.73	74.9	23.98	71	24.56	70.6
AH010915.7	D	16.44	25.22	76.6	25.61	70.8	24.44	75.1	24.67	70.8	27	86.2
AH090915.1	D	17.02	25.48	76.6	25.23	70.8	30.21	73.6	28.95	70.8	31.22	83.6
AH010915.6	D	17.75	27.82	76.6	30.58	70.8	26.01	75.1	27.15	70.8	28.03	70.9
AH010915.4	D	19.98	29.3	76.6	28.22	70.8	27.95	75.1	26.92	71.4	29.46	70.9
AH010915.5	D	18.91	29.22	77.2	29.81	73.8	25.71	75.1	26.15	71.7	39.33	84.3
AH070915.12	D	20.18	29.29	77.2	29.68	71.1	28.14	75.7	26.5	71.7	34.04	85.6
AH070915.11	D	17.55	25.24	77.2	24.7	71.1	27.4	75.7	23.75	71.7	35.14	86.2
AH080915.27	D	18.65	29.63	77.2	29.34	70.8	26.39	75.4	26.08	71.4	U	81.1
AH080915.29	D	18.05	27.34	76.9	26.27	70.8	27.11	75.4	25.29	71.4	31.12	70.9
AH090915.3	D	19.01	28.21	76.9	27.78	75.3	27.64	75.1	25.36	71.1	28.41	70.9
Negative Control	-	-	32.29	76.9	40.03	72.9	26.96	75.1	25.85	71.1	32.59	70.9

3.3.2 SRY gene (SRYhmg primers)

The SRYhmg primer set was designed by Sanchez et al. (1996) to amplify a 202 bp section of the SRY gene in male mammals. Of the eight *R. hipposideros* individuals tested with these primers, three (A, G and H) produced bright bands of the appropriate size (Fig. 3.8). However, the other samples (B- F) also produced a number of fainter bands of varying size. Products from two of the samples which showed amplification (A and G) were sequenced, resulting in a sequence of 204 bp (Fig. 3.9). This sequence was found to be homologous to published SRY sequences (Table 3.6). A neighbour joining tree was constructed using this sequence and the most similar published sequences (Fig. 3.10).

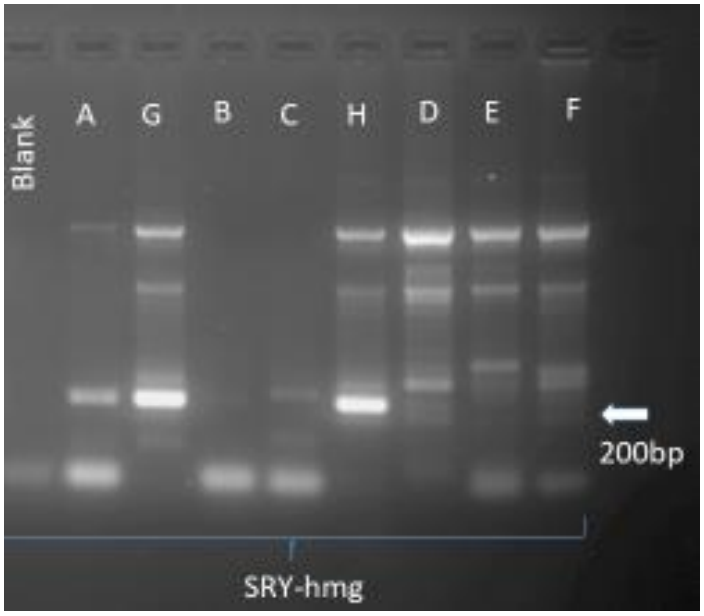

Figure 3.8: Gel image of products of PCR reaction of *R. hipposideros* individuals with SRY-hmg primer set, using the Touchdown protocol as per Hellborg and Ellegren (2003). 4 µl of each product was inserted per well, on a 2% agarose gel.

TGTCAAGCGCCCCATGAATGCATTCATGGTGTGGTCTCGCGATCAAAGGCGCAAAGTGGC 60

TCTAGAAAATCCCAAAATGCACAACTCAGAGATCAGCAAGCAGCTAGGAAGCCAGTGGAA 120

AATGCTGACGGAAGCCGAAAAGTGCCCATTCTTCGAGGAGGCACAGAAGCTACGCGCCTT 180

GCACAGAGAGAAATACCCAAACTA 204

Figure 3.9: SRY sequence obtained for *R. hipposideros* using the SRY-hmg primer set. The position of real-time PCR primers designed based on this sequence are highlighted in blue (RhipSRYhmg-F/R).

Table 3.6: BLAST search results for *R. hipposideros* SRY sequences derived from the SRYhmg primers.

SRY product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Equus kiang*	Tibetan wild ass	SRY	97%	88%
2	*Equus asinus*	Donkey	SRY	97%	88%
3	*Equus przewalski*	Przewalski's horse	SRY	97%	88%
4	*Canis lupus*	Wolf	SRY	96%	87%
5	*Vulpes vulpes*	Red fox	SRY	96%	87%
6	*Vulpes lagopus*	Arctic fox	SRY	96%	86%
7	*Cryptotis mexicana*	Mexican small-eared shrew	SRY	96%	86%
8	*Nyctereutes procyonoides*	Raccoon dog	SRY	96%	85%
9	*Microtus cabrerae*	Cabrera's vole	SRY	99%	80%
10	*Cavia porcellus*	Guinea pig	SRY	47%	85%

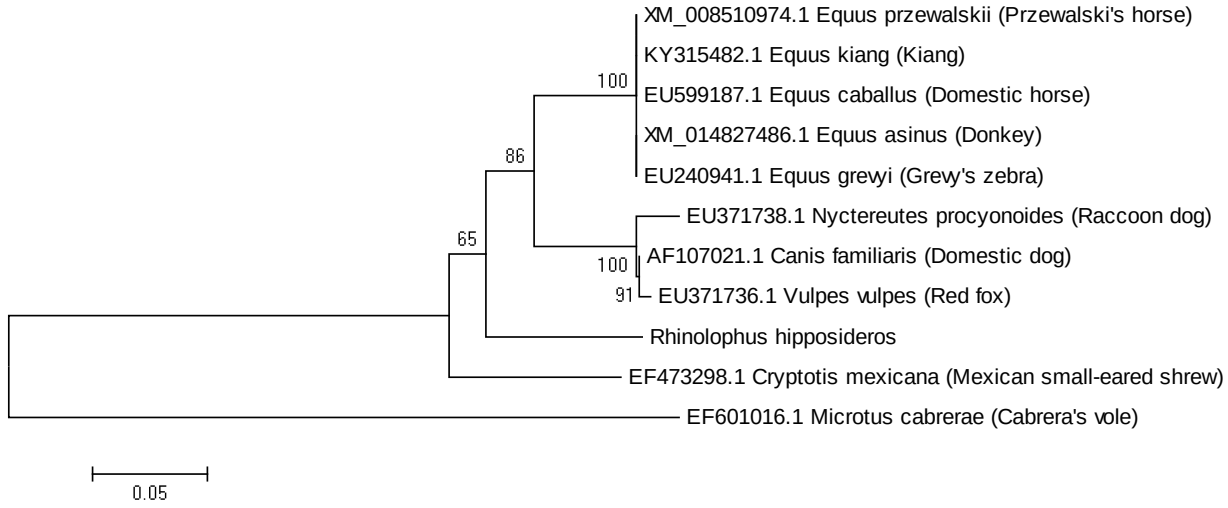

Figure 3.10: Neighbour joining trees of SRY sequences of *R. hipposideros* and the most similar published sequences on GenBank. Neighbour joining tree were constructed using 1000 bootstraps and genetic distance was computed using the P-distance method (Nei and Kumar, 2000).

A single primer set, RhipSRYhmg-F/R, was designed to target a section of the *R. hipposideros* SRY sequence (Fig. 3.9; Table 3.16). This primer set was tested simultaneously with the RhipZFX3F/R primer pair to act as an internal control. This primer successfully amplified the tissue samples previously identified as male based on the production of male-specific bands by the SRY conventional PCR primers, and also identified three faecal DNA samples as

originating from males. Ct values were also seen to increase as DNA template decreased using a dilution series (Fig. 3.11; Table 3.7).

Table 3.7: Results of real-time PCR sex typing assays designed for *R. hipposideros*, based on sequence data obtained using the SRY-hmg primer set. Ct values for samples identified as male are highlighted in bold type.

Individual/Faecal Sample	Sample Type	RhipCytb Ct	RhipZFX3		RhipSRYhmg	
			Ct	Tm	Ct	Tm
A	T	15.82	**23.62**	70.8	**24.09**	76.6
B	T	14.92	31.16	70.8	35.77	78.1
C	T	15.27	26.33	70.8	35.17	79.5
D	T	14.42	22.19	70.8	32.53	79.5
E	T	14.33	22.35	70.8	31.85	77.1
F	T	14.23	22.9	70.8	33.55	79.3
G	T	14.88	**23.43**	70.8	**24.48**	77.1
H	T	15.7	**24.09**	70.8	**24.27**	77.5
AH090915.1	D	17.02	25.07	70.1	33.08	75.8
AH010915.6	D	17.75	**28.42**	70.1	**27.74**	76.5
AH010915.4	D	19.98	29.03	70.8	32.8	77.1
AH010915.5	D	18.91	**30.38**	70.4	**29.08**	77.5
AH070915.12	D	20.18	31.6	70.8	42.49	74.4
AH070915.11	D	17.55	25.38	70.8	39.4	80.3
AH080915.27	D	18.65	29.99	71.1	39.73	72.4
AH080915.29	D	18.05	26.77	71.1	42.51	74.4
AH090915.3	D	19.01	28.11	71.1	42.36	75.1
Negative Control	-	-	44.76	72.1	35.47	74.2

Non-specific amplification was also produced in female samples and in negative controls, but this was distinguishable from male-specific amplification by melt curve analysis. Male samples displayed a dissociation curve with a Tm of 77.1 ± 0.4°C, while those of female or negative controls displayed widely varying melting temperatures and multiple peaks in some cases (Fig. 3.12; Table 3.7). In addition, a difference of at least 7 cycles was seen between the highest Ct value produced by a male sample and the lowest Ct value produced by a female sample or negative control. Male samples showed simultaneous amplification of both the ZFX and SRYhmg primers, to within one cycle (Table 3.8).

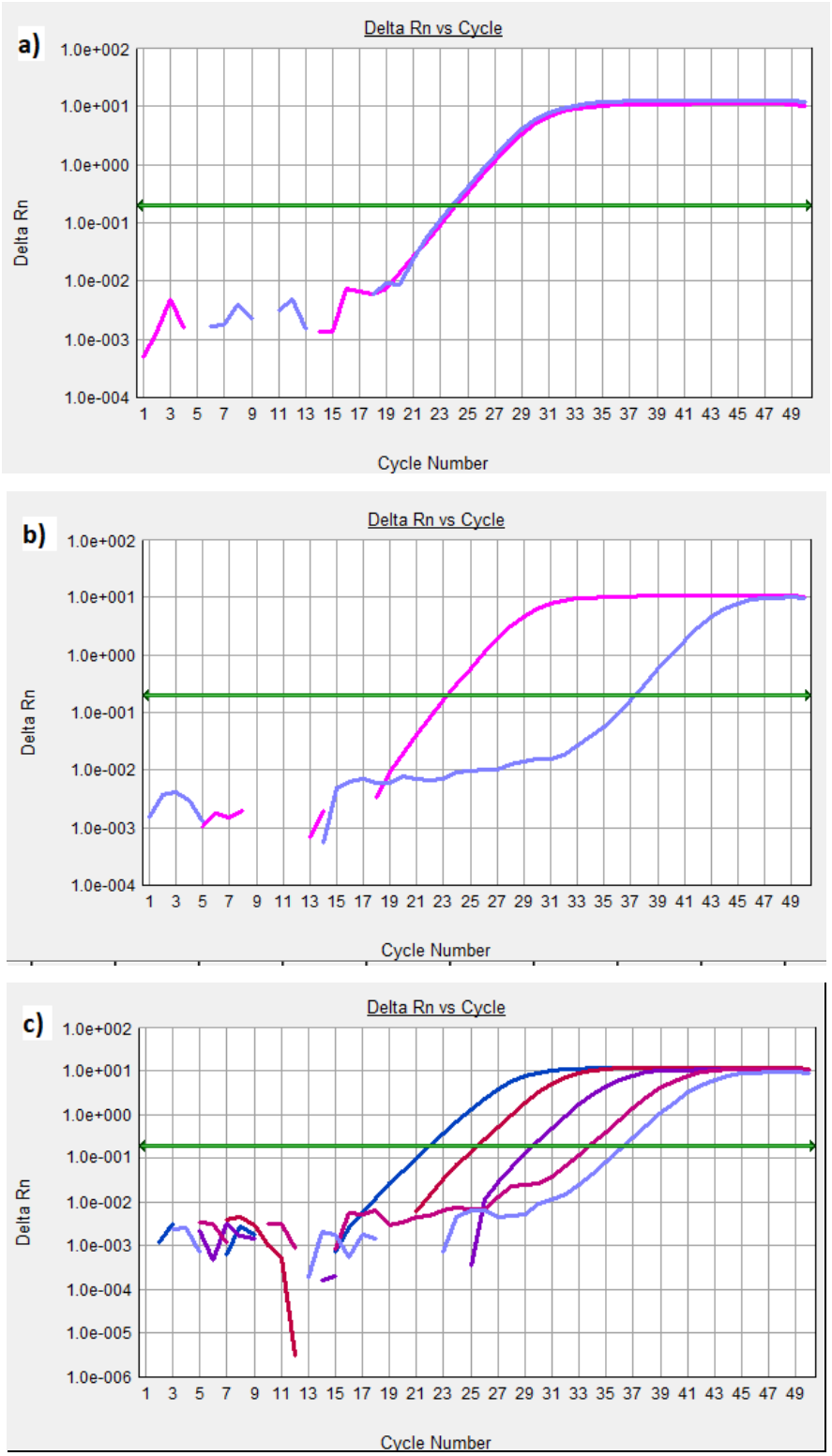

Figure 3.11: Real-time PCR amplification of *R. hipposideros* tissue DNA from a male (a) and female (b), using the RhipZFX-F/R3 and the RhipSRYhmg-F/R primer sets. An example of a dilution series of a tissue sample amplified by the RhipSRYhmg-F/R primer set is shown at (c), with a starting concentration of 10ng/µl of DNA. Pink = ZFX assay amplification, blue = SRY-hmg assay amplification.

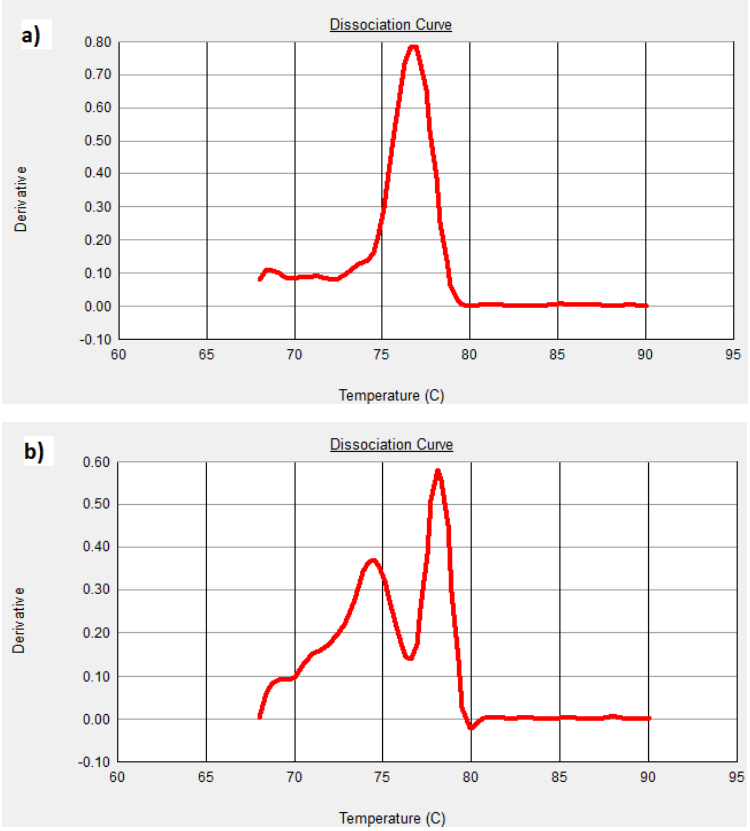

Figure 3.12: Melt curves of real-time PCR products of a male DNA sample (a) and a female sample (b).

Table 3.8: Results of real-time PCR amplification of a dilution series of a tissue DNA sample from a male *R. hipposideros*, using the RhipZFX-F/R3 and RhipSRYhmg-F/R primer sets. The starting concentration of the dilution series was 10ng/µl.

	Ct	
Dilution factor	**RhipZFX3**	**RhipSRYhmg**
1e+1	22.9	22
1e+0	25.2	25.7
1e-1	29.9	29.8
1e-2	32.1	32.3

3.3.3 DBY gene (DBY3 primers)

A primer set targeting intron 3 of the DBY gene was designed by Hellborg and Ellegren (2003), which was found to amplify a 300-700 bp product in a range of mammal species. As mentioned in section 3.1, this primer set was used by Dool (2010) to identify male *R. hipposideros* from unsexed tissue samples. When *R. hipposideros* individuals A-H were tested using this primer set a 400 bp product was amplified for individuals A, G and H (Fig. 3.13), from which a 360 bp sequence was obtained (Fig. 3.14). When subjected to BLAST analysis, this sequence showed homology to published Y chromosome sequences, including DBY3 sequences (Table 3.9).

A single real-time PCR primer set, RhipDBY3F/R, was designed to target a section of the *R. hipposideros* DBY3 sequence (Fig. 3.14; Table 3.16). However, in a real-time PCR reaction using the tissue DNA samples from the eight individual bats and faecal DNA samples, the primer set failed to identify male individuals, with either late amplification or none at all (Table 3.10).

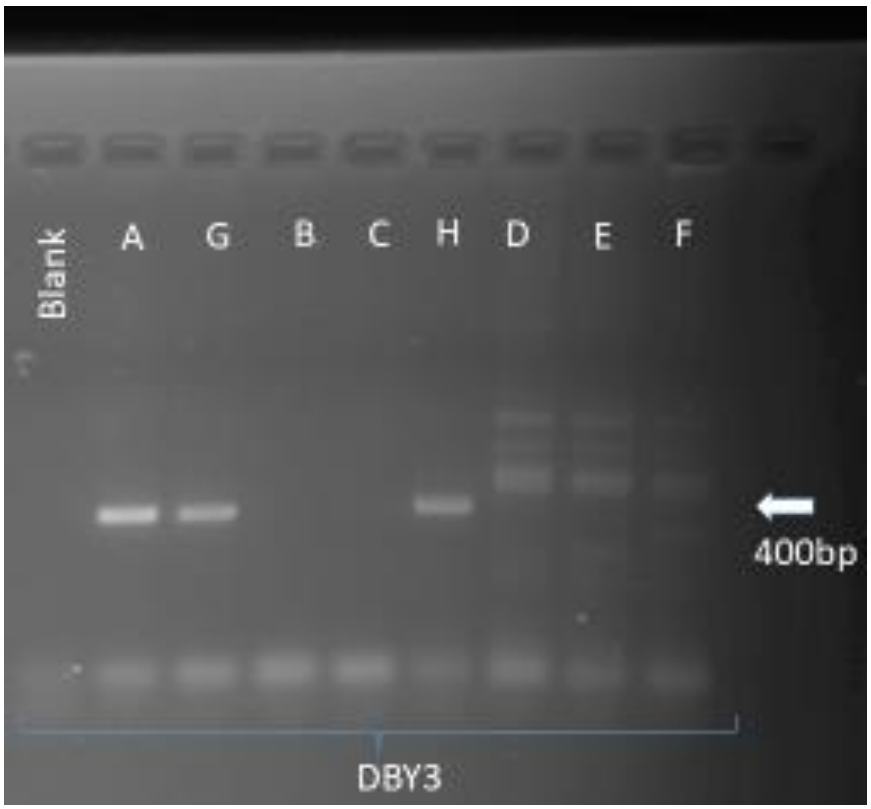

Figure 3.13: Gel image of products of PCR reaction of *R. hipposideros* individuals with DBY3 primer set. 4 µl of each product was inserted per well, on a 2% agarose gel.

ACTCTCTGTCTGCGCACACCCTTCAGAGGAGAAACACTGAGCCAAGTCAAGAGGGTTTAG 60

TGGAGGTAATACTGATTTTCCTCTTTTGTACTTTGGAAAGGGCTTTTTCCCCCCTTAATC 120

TTTTTATTTTTAAGTCTCAATGGGGTTTCTTTATCCTTACTCTTTTTTAATGCATAATTT 180

ATTTAATAAATTTTATTTCCAAAGAACACAAATCCAGTACAGATTTATTTTATTGGTCTT 240

ATAATCAAAGTTCTGGAAAAAGAAAGGAAAAGATACTTAGAGTAGCTGACCTAATTTCTC 300

TTTTCAAACATTTCTTTACAGGTGGCTATGGAGGCTTCTACAATAATGATGGTTATGGAA 360

Figure 3.14: DBY intron 3 sequence obtained for *R. hipposideros* using the DBY3 primer set. The position of real-time PCR primers designed based on this sequence are highlighted in blue (RhipDBY3-F/R).

Table 3.9: BLAST search results for *R. hipposideros* SRY sequences derived from the DBY3 primers.

DBY3 product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Felis catus*	Domestic cat	Y Chr.	53%	75%
2	*Homo sapiens*	Human	Y Chr.	55%	75%
3	*Canis lupus familiaris*	Dog	Y Chr.	56%	73%
4	*Pan troglodytes*	Common chimpanzee	Y Chr.	55%	75%
5	*Sorex alpinus*	Alpine shrew	DBY	19%	90%
6	*Sorex samniticus*	Apennine shrew	DBY	19%	90%
7	*Sorex coronatus*	Millet's shrew	DBY	19%	90%
8	*Sorex granarius*	Iberian shrew	DBY	19%	90%
9	*Sorex araneus*	Common shrew	DBY	19%	90%
10	*Callithrix jacchus*	Common marmoset	Y Chr.	54%	72%

Table 3.10: Results of real-time PCR sex typing assays designed for *R. hipposideros*, based on sequence data obtained using the DBY3 primer set. Negative amplification is indicated by "U" (for "undetermined").

Individual/ faecal sample	Sample type	RhipCytb Ct	RhipZFX3		RhipDBY3	
			Ct	Tm	Ct	Tm
A	T	15.82	22.31	70.1	U	76.8
B	T	14.92	31.87	70.1	U	85.7
C	T	15.27	25.41	70.8	49.2	69.9
D	T	14.42	21.56	70.8	38.52	69.9
E	T	14.33	23.95	70.4	U	78.8
F	T	14.23	22.46	70.8	47.5	76.4
G	T	14.88	21.46	70.1	U	74.5
H	T	15.7	23.49	70.4	47.3	76.1
AH090915.1	D	17.02	25.07	70.1	48.84	70.8
AH010915.6	D	17.75	28.42	70.1	U	75.8
AH010915.4	D	19.98	29.03	70.8	U	71.7
AH010915.5	D	18.91	30.38	70.4	U	77.8
AH070915.12	D	20.18	31.6	70.8	U	82
AH070915.11	D	17.55	25.38	70.8	U	77.5
AH080915.27	D	18.65	29.99	71.1	U	80.9
AH080915.29	D	18.05	26.77	71.1	U	82
AH090915.3	D	19.01	28.11	71.1	U	75.5
Negative Control	-	-	44.76	72.1	U	78.1

3.3.4 DBY gene (DBY7 primers)

This primer set was designed to target intron 7 of the DBY gene by Hellborg and Ellegren (2003), resulting in male-specific products varying in size from 300-750 bp in a range of mammal species. PCR reactions carried out using all eight *R. hipposideros* tissue DNA samples resulted in the production of 300 bp bands in individuals A, G and H, as well as fainter non-specific bands in the remaining individuals (Fig. 3.15). When the PCR products from the three male samples were sequenced, a 248 bp consensus sequence was obtained (Fig. 3.16). When subjected to BLAST analysis, this sequence showed homology with published DBY7 sequences (Table 3.11). A neighbour joining tree was constructed using this sequence and the most similar published sequences (Fig. 3.17).

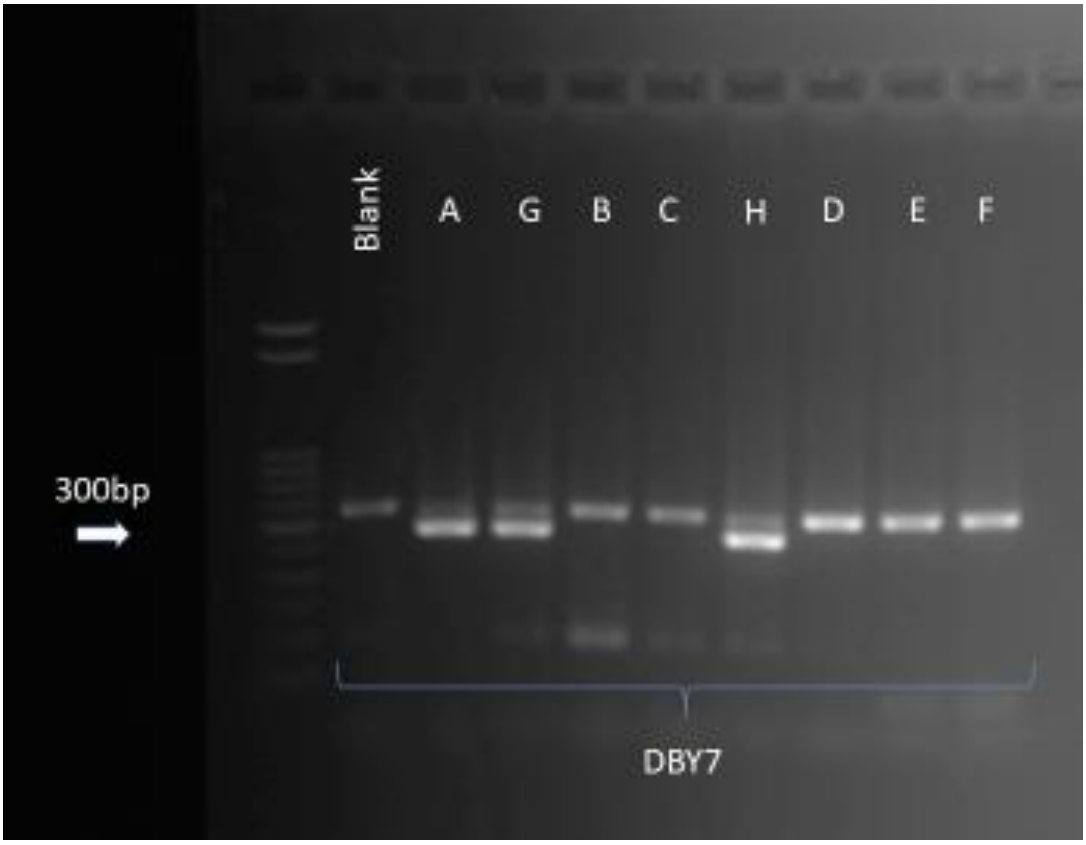

Figure 3.15: Gel image of products of PCR reaction of *R. hipposideros* individuals with DBY7 primer. 4 µl of each product was inserted per well, on a 2% agarose gel.

TTATAAAACGTTTTACAAATATCTGTGGGCACATTTCTTTTCAATCTATTTCTCCAGAAG 60

ATCTATATGTATTTCTTAAATTACAAAGGGAATTTTGATATTATGATTTTTAAAAATAAT 120

AAAAGTTTTGGGTCTTTGACTCTAATTTATAAATTAAATACTTAAATTTCTTAGGAAAAT 180

GGACGATATGGACGCCGTAAACAATACCCAATCTCCTTGGTTTTAGCCCCAACAAGAGAA 240

TTGGCTGA 248

Figure 3.16: DBY intron 7 sequence obtained for *R. hipposideros* using the DBY7 primer set. The position of real-time PCR primers designed based on this sequence are highlighted in blue (RhipDBY7-F/R).

Table 3.11: BLAST search results for *R. hipposideros* DBY sequences derived from the DBY7 primers.

DBY7 product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Rhinolophus affinis*	Intermediate horseshoe bat	DBY	84%	91%
2	*Rhinolophus alcyone*	Halcyon horseshoe bat	DBY	84%	89%
3	*Hipposideros ruber*	Noack's roundleaf bat	DBY	84%	85%
4	*Equus asinus*	Donkey	DBY	86%	82%
5	*Equus caballus*	Domestic horse	DBY	86%	82%
6	*Tapirus indicus*	Malayan tapir	DBY	72%	84%
7	*Mesoplodon perrini*	Perrin's beaked whale	DBY	72%	84%
8	*Mesoplodon grayi*	Gray's beaked whale	DBY	72%	84%
9	*Cervus eldi*	Eld's deer	DBY	70%	83%
10	*Bos taurus*	Domestic cow	Y Chr.	72%	82%

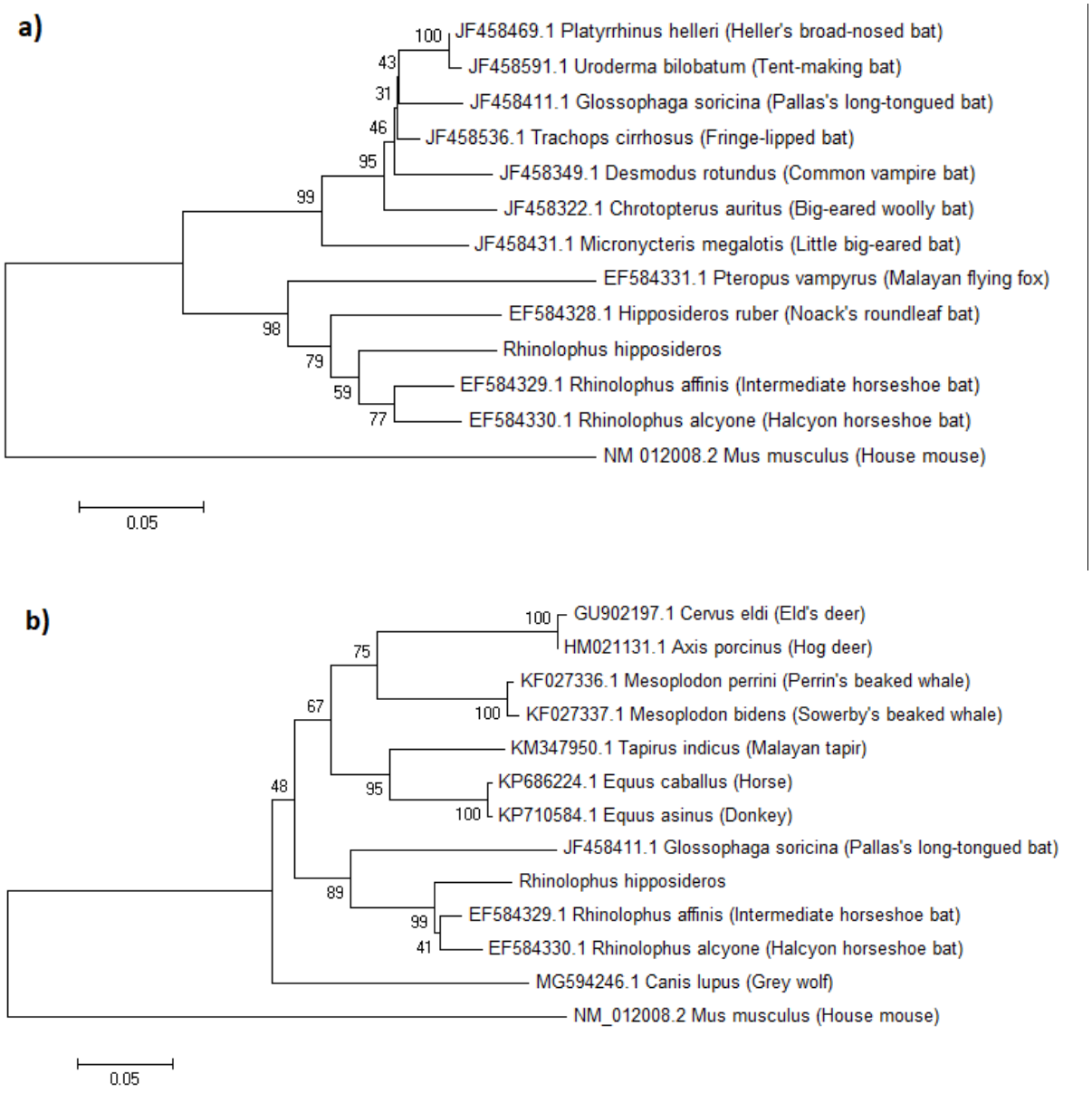

Figure 3.17: Neighbour-joining tree of DBY7 sequences from (a) a variety of bat species and (b) a selection of other closely related mammal groups. Neighbour-joining tree was constructed using 1000 bootstraps and genetic distance was computed using the P-distance method (Nei and Kumar, 2000).

A single primer set, RhipDBY7-F/R, was designed to target a section of the *R. hipposideros* DBY7 sequence (Fig. 3.16; Table 3.16). This primer set was tested simultaneously with the RhipZFX3F/R primer pair to act as an internal control. This primer successfully amplified the tissue samples previously identified as male based on the production of male-specific bands by the DBY7 conventional PCR primers and also identified three faecal DNA samples as

originating from males. Ct values were also seen to increase as DNA template decreased using a dilution series (Fig. 3.18; Table 3.12).

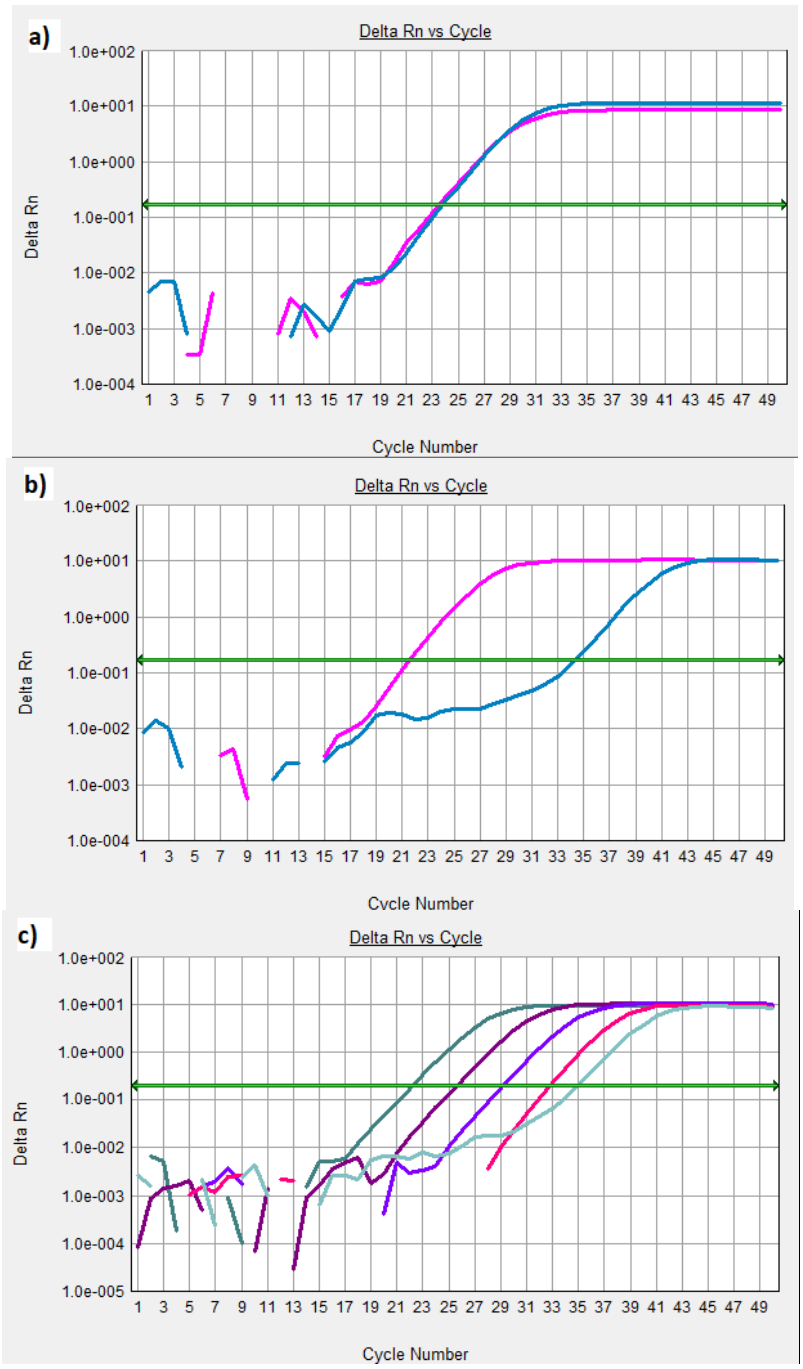

Figure 3.18: Real-time PCR amplification of *R. hipposideros* tissue DNA from a male (a) and female (b), using the RhipZFX-F/R3 and the RhipDBY7-F/R primer sets. An example of a dilution series of a tissue sample amplified by the RhipDBY7-F/R primer set is shown at (c), with a starting concentration of 10ng/µl of DNA. Pink = ZFX assay amplification, blue = DBY7 assay amplification.

Table 3.12: Results of real-time PCR sex typing assays designed for *R. hipposideros*, based on sequence data obtained using the DBY7 primer set. Ct values for samples identified as male are highlighted in bold type.

Individual/ faecal sample	Sample type	RhipCytb Ct	RhipZFX3		RhipDBY7	
			Ct	Tm	Ct	Tm
A	T	15.82	**22.31**	70.1	**24.02**	72.7
B	T	14.92	31.87	70.1	35.29	73.7
C	T	15.27	25.41	70.8	33.01	85.3
D	T	14.42	21.56	70.8	34.37	78.8
E	T	14.33	23.95	70.4	32.64	73.3
F	T	14.23	22.46	70.8	34.34	73
G	T	14.88	**21.46**	70.1	**20.77**	72.7
H	T	15.7	**23.49**	70.4	**23.81**	74
AH090915.1	D	17.02	25.07	70.1	38.13	73.8
AH010915.6	D	17.75	**28.42**	70.1	**25.18**	73.1
AH010915.4	D	19.98	29.03	70.8	31.39	73.4
AH010915.5	D	18.91	**30.38**	70.4	**29.87**	73.4
AH070915.12	D	20.18	31.6	70.8	38.67	74.4
AH070915.11	D	17.55	25.38	70.8	34.59	73.4
AH080915.27	D	18.65	29.99	71.1	32.37	84.9
AH080915.29	D	18.05	26.77	71.1	32.22	84.9
AH090915.3	D	19.01	28.11	71.1	31.23	73.8
Negative Control	-	-	44.76	72.1	38.09	76

Non-specific amplification was also produced in female samples and in negative controls, but this was distinguishable from male-specific amplification by several means. A difference of nine cycles was noted between the highest Ct value produced by a male sample and the lowest Ct value produced by a female sample or negative control. Male samples showed simultaneous amplification of both the ZFX and DBY7 primers, to within one cycle (Table 3.13). In addition, melt-curve analysis showed that PCR products of male individuals displayed a curve with a Tm of 74.4 ± 0.5°C. In contrast, products of non-specific amplification displayed curves with multiple peaks (Fig. 3.19).

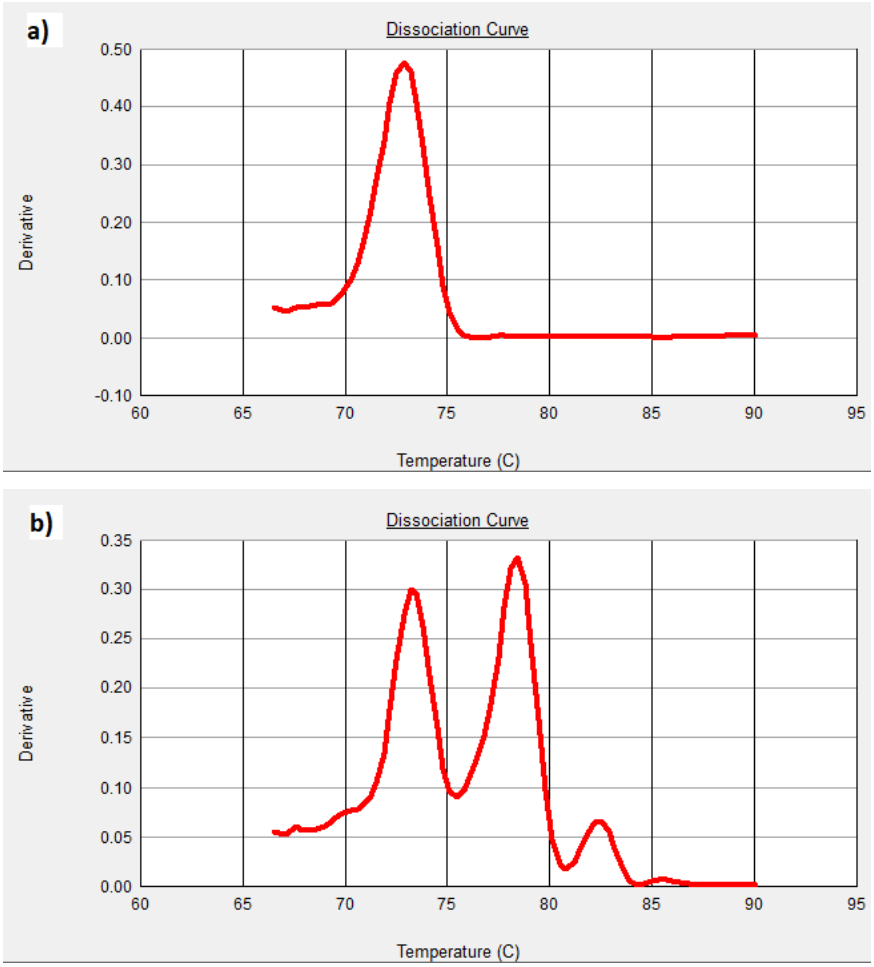

Figure 3.19: Melt curves of real-time PCR products of a male DNA sample (a) and a female sample (b).

Table 3.13: Results of real-time PCR amplification of a dilution series of a tissue DNA sample from a male *R. hipposideros*, using the RhipZFX-F/R3 and RhipDBY7-F/R primer sets. The starting concentration of the dilution series was 10ng/µl.

	Ct	
Dilution factor	**RhipZFX3**	**RhipDBY7**
1e+1	22.9	22.2
1e+0	25.2	25.5
1e-1	29.9	29.1
1e-2	32.1	33

3.3.5 DBY gene (DBY8 primers)

A primer set targeting intron 8 of the DBY gene was designed by Hellborg and Ellegren (2003), which was found to amplify a 200 bp product in a wide range of mammal species. *R. hipposideros* individuals A-H were tested with this primer set, resulting in an approximately 180 bp product in individuals A, G and H when visualised using gel electrophoresis (Fig. 3.20). A 132 bp sequence was obtained from these individuals (Fig. 3.21).

DNA BLAST analysis showed that this sequence was closely homologous to published DBY8 sequences from other mammal species (Table 3.14). This sequence showed closest identity to a recently published *R. hipposideros* DBY8 sequence (Zarzoso-Lacoste, 2018).

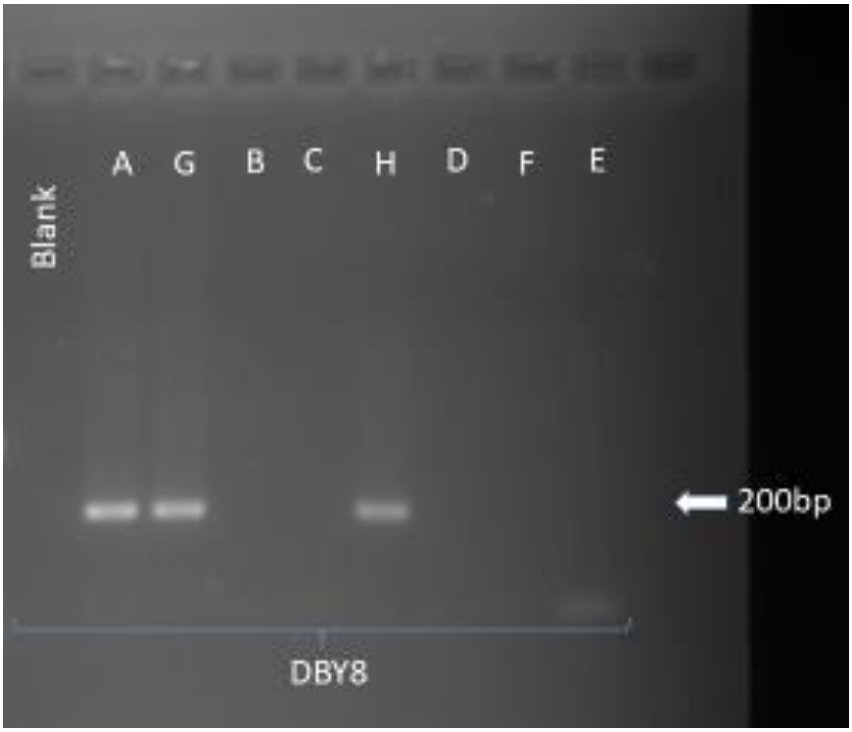

Figure 3.20: Gel image of products of PCR reaction of *R. hipposideros* individuals with the DBY8 primer. 4 µl of each product was inserted per well, on a 2% agarose gel.

GTAATATACATTTTACTGAATATTGGCATTTTTATTGTTCTAATGCTAATTTTATGACCA 60

CCTAATACATTTTTTGTTTATAGTTTTCATACCGCTCTAGAGTTCGCCCTTGTGTAGTCT 120

ATGGTGGTGCTG 132

Figure 3.21: DBY intron 8 sequence obtained for *R. hipposideros* using the DBY8 primer set.

Table 3.14: BLAST search results for *R. hipposideros* SRY sequences derived from the DBY8 primers.

DBY8 product					
	Species	Common name	Gene target	Query coverage	Identity
1	*Rhinolophus hipposideros*	Lesser horseshoe bat	DBY	98%	99%
2	*Lobodon carcinophaga*	Crab-eater seal	DBY	100%	86%
3	*Ommatophoca rossii*	Ross seal	DBY	100%	86%
4	*Leptonychotes weddellii*	Weddell seal	DBY	96%	86%
5	*Cervus elaphus*	Red deer	DBY	100%	83%
6	*Cervus nippon*	Sika deer	DBY	100%	81%
7	*Canis lupus familiaris*	Dog	Y Chr.	93%	82%
8	*Lepus europeaus*	Brown hare	DBY	61%	90%
9	*Phacochoerus africanus*	Common warthog	DBY	97%	80%
10	*Callithrix jacchus*	Common marmoset	Y Chr.	98%	78%

An attempt was made to design real-time PCR primers based on this sequence, but as no suitable primers could be found due to the short length and very low GC content of the sequence, this marker was not examined further.

3.4 Discussion

The aim of this chapter was to design a sex determination assay for *Rhinolophus hipposideros* suitable for use with non-invasive samples, by means of testing previously published sex determining markers. Clear results were obtained from four sex determination genes: ZFX, ZFY, SRY and DBY. The DNA sequence data obtained gave consistent sex typing results for each *R. hipposideros* individuals from which tissue samples had been obtained (Table 3.15).

Table 3.15: Inferred sex of *R. hipposideros* individuals based on published conventional PCR sex typing primers used (males marked M, females marked F).

Primer set	**Sex of *R. hipposideros* individual**							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
LGL331/LGL335	M	F	F	F	F	F	M	M
SRY-hmg	M	F	F	F	F	F	M	M
DBY3	M	F	F	F	F	F	M	M
DBY7	M	F	F	F	F	F	M	M
DBY8	M	F	F	F	F	F	M	M

The DNA sequences obtained using the LGL331/LGL335 and RhipLGLy primer sets showed homology to published sequences from other mammal species of the ZFX and ZFY genes, respectively. Sequence data was also successfully obtained using primers targeting the SRY gene and introns 3, 7 and 8 of the DBY gene, all of which showed homology to previously published sequences of these genes from other mammal species when subjected to BLAST analysis. In particular, the sequences obtained for the DBY7 and DBY8 markers were very similar to other *Rhinolophus* species, or identical to published *R. hipposideros* sequences. Several of these markers have previously been successfully used to identify male *R. hipposideros*, namely the SRYhmg, DBY3 and DBY8 regions.

Phylogenetic analysis was carried out using novel *R. hipposideros* DNA sequences from four regions of the sex chromosomes, including the ZFX intron from the X chromosome, and the ZFY intron, SRY HMG box and DBY7 intron regions of the Y chromosome. The phylogenetic trees created using these sequences showed differing patterns, which may be partially due to the set of species for which DNA sequences were available on GenBank, which varied between the different markers used. The most interesting patterns emerged in the phylogenetic trees

created using the DBY7 sequences, for which a wide range of other sequences were available for comparison (Fig. 3.17). A phylogenetic tree was firstly created using only sequences from other bat species from a range of families from the "microbat" and "megabat" groups (Fig. 3.17a). Interestingly, this tree showed that *Rhinolophus hipposideros* and a number of other related microbat species from the Rhinolophid and Hipposiderid families are more closely related to megabat species (i.e. fruit bats and flying foxes) than they are to other microbat families. This pattern matched the phylogenetic taxonomy demonstrated by Teeling et al. (2005), whereby bats are now classified into the suborders Yinpterochiroptera (megabats and horseshoe bats and relatives) and Yangochiroptera (all other microbats), even though the DBY7 gene has not been previously used for an overall examination of the phylogeny of bats as a whole, although it has been used for phylogenetic studies of some bat families (Lim et al., 2007; Clare, 2011).

When the *R. hipposideros* DBY sequence was compared with a wider variety of mammal species, it showed the greatest similarity to species belonging to the orders Artiodactyla (e.g. deer and cattle), Cetacea (whales and dolphins), Perissodactyla (e.g. tapirs and horses) and Carnivora. Again, this reflects the taxonomic position of the order Chiroptera, which has been shown to belong a diverse clade of mammals called the Laurasiatheria, including the Chiroptera, Artiodactyla, Cetacea, Perissodactyla, Carnivores, Pholidota (pangolins) and Eulipotyphla (hedgehogs and shrews), based on phylogenetic analysis (Tsagkogeorga et al., 2013). However, the phylogenetic tree shown in Fig. 3.17b showed bats to be more closely related to artiodactyls, cetaceans and perissodactyls than carnivores. This contrasts with published phylogenetic analyses which place bats as one of the most distantly related groups to the other Laurasiatherians, after the Eulipotyphla (Tsagkogeorga et al., 2013). This discordance between the DBY7 phylogenetic tree and published studies may illustrate "gene tree discordance", but it also possible that using longer sequences, or a more carefully selected set of sequences from a wider variety of mammal orders, would have shown the currently accepted pattern of taxonomy. Interestingly, the phylogenetic tree created based on the SRY HMG box region sequence (Fig. 3.10) displayed the a pattern most similar to the accepted Laurasiatherian taxonomy, showing carnivores and perissodactyl to be closely related, with bats and Eulipotyphlans (represented by *R. hipposideros* and a shrew species, respectively) being more distantly related groups.

The phylogenetic trees based on the *R. hipposideros* ZFX intron and ZFY intron sequences also showed patterns which were more difficult to explain. While the sequences were most

similar to other published ZFX and ZFY sequences, the trees showed *R. hipposideros* to be the most distantly related of all groups, including those intended to be outgroups, even though all of the other sequences used broadly showed the currently accepted taxonomy of the mammal orders which they belonged to (Fig.s 3.3 and 3.6). The trees created for these genes were somewhat limited in the range of other species for which sequences were available, and it should also be noted that the sequences obtained in this study were relatively short, both of which may not have given enough power for MEGA to differentiate between distantly related groups. Overall, the phylogenetic trees created using the sequence data obtained in this study support the close relationship of *R. hipposideros* to Laurasiatherian mammal species, but also highlight the need to depend on a number of genetic markers in phylogenetic studies of distantly related species in order to arrive at a consensus phylogeny.

Based on the sequence data obtained for *Rhinolophus hipposideros*, eight real-time PCR primer sets were designed in order to develop a sex determination assay for this species (Table 3.16). These included two primer sets which targeted sections of the ZFX gene in order to act as an internal control, and nine primer, or primer and probe, sets to target sections of the ZFY, SRY and DBY genes in order to identify male individuals. The effort to design a sex determination assay proved difficult, as some of the primer sets designed did not appear to be specific to the target sequences, and non-specific amplification was encountered in all primer sets to some extent. Given that such amplification was even seen in negative controls, this implies that primer-dimer formation may have been an issue, or that the primer sets exhibited cross-reactivity for other DNA sequences which may have been present as contamination. Contaminants may have included human or microbial DNA, or DNA from other mammal species which have been studied in the same laboratory including a range of bat species, mustelids, small mammals, squirrels and hedgehogs.

One of the real-time primer sets, RhipZFX3, was found to successfully amplify the targeted sequence, exhibiting a low level of non-specific amplification which was found to be distinguishable by melt-curve analysis. This primer set was thus selected for use as an internal control and subsequently used for further testing of male-specific primers.

Although the conventional PCR primer, RhipLGLy, successfully amplified a targeted sequence of the ZFY gene, none of the real-time PCR primers and probes based on this sequence were able to correctly identify male individuals. Of the primer sets designed to target the SRY and DBY genes, one also failed to identify male individuals. However, RhipSRYhmg and

RhipDBY7 both accurately identified the tissue DNA samples from male individuals, and also identified the same two faecal DNA samples as having originated from males (Table 3.17).

Table 3.16: List of conventional and real-time PCR primers and probes designed for the se typing of R. hipposideros.

Target gene	Primer	qPCR Primer/ Probe	qPCR Primer/Probe sequence (5'-3')
ZFX	LGL331/LGL335	RhipZFX-F/R2	F: ACTATTGGACAAGCACTGCTAAG R: ACCGGAGGGAGTGAGATTGA
ZFX	LGL331/LGL335	RhipZFX-F/R3	F: CACAATTTCTGCTTTGGTTATTCCA R: CAGCAGGACAGGGACTTCAG
ZFY	LGL331/LGL335	Rhip-LGLy-F/R	F: GCT AAG AAG GAA AAG GAG TGT GA R: AGA CAG TAC CAA ACA GGT GAG G
ZFY	LGL331/LGL335	RhipZFY-F/R2	F: AAGCTACACATGGTCTAACAGCTA R: GGGCACATGAGTTCCATAGCA
ZFY	LGL331/LGL335	RhipZFY-F/R3	F: GCCCAATGAACAACCACTCC R: TGGAAATGACAGTAAAGCCCTAAA
ZFY	LGL331/LGL335	RhipZFY-F/R4	F: CATTATGACAGAACCAATCTCACAAAG R: ATTGCCTTGTTAAACGATTTGCAT
SRY	SRY HMG F/R	RhipSRYhmg-F/R	F: CTCAGAGATCAGCAAGCAGCTA R: GCCTCCTCGAAGAATGGGCAC
DBY3	DBY3F/R	RhipDBY3-F/R	F: CACACCCTTCAGAGGAGAAACAC R: CAAAGTACAAAAGAGGAAAATCAGTATTACC
DBY7	DBY7F/R	RhipDBY7-F/R	F: AATGGACGATATGGACGCCG R: CAGCCAATTCTCTTGTTGGGG

Table 3.17: Results of the two successful real-time PCR sex typing assays designed to differentiate between male and female *R. hipposideros* individuals, RhipSRYhmg and RhipDBY7 (RhipZFX3 not shown as it used only as an internal control). Results show the sex assigned to the *R. hipposideros* individuals and faecal DNA samples tested.

Individual/faecal sample	Sample type	Sex of individual/faecal sample	
		RhipSRYhmg	RhipDBY7
A	T	M	M
B	T	F	F
C	T	F	F
D	T	F	F
E	T	F	F
F	T	F	F
G	T	M	M
H	T	M	M
AH090915.1	D	F	F
AH010915.6	D	M	M
AH010915.4	D	F	F
AH010915.5	D	M	M
AH070915.12	D	F	F
AH070915.11	D	F	F
AH080915.27	D	F	F
AH080915.29	D	F	F
AH090915.3	D	F	F

Therefore, a real-time PCR sex determination assay has been successfully developed, comprising an internal control targeting the ZFX gene and two primers targeting separate male-specific markers. A similar assay was recently developed by Zarzoso-Lacoste et al. (2018), but the sex typing assay published, DDX3X/Y-Mam, is a conventional PCR primer set which requires DNA sequencing and fragment size analysis of the PCR products to actually identify the sample being tested as a male or female, which requires a significant amount of post-PCR processing and data analysis. In contrast, the set of real-time PCR assays designed in this study has several advantages over the Zarzoso-Lacoste et al.'s (2018) method. Real-time PCR has been noted as being the most sensitive form of PCR for the amplification of low quantities of DNA, which is a common issue with non-invasively collected samples, and the SYBR Green I chemistry used in this study is also highly reliable (Beja-Pereira et al., 2009). It also requires no post-PCR processing, eliminating the potential for contamination of the samples being tested and reducing the time and cost involved in DNA analysis and thus increasing sample throughput (Beja-Pereira et al., 2009; Mullins et al., 2010; O'Neill et al., 2013). In addition, the process of data analysis for the assays designed in this study provides a simple system of identifying positive amplification based on Ct values, which is further bolstered by the use of

melt curve analysis, whereby the unique melting temperature (Tm) of the target product can be distinguished from potential non-specific amplification or primer-dimers (Bio-Rad, 2006). Thus, the assay designed in this study represents an improved method of sex typing for the lesser horseshoe bat, which is particularly well suited to working with non-invasively collected DNA samples, and will provide the advantage in increasing throughput of samples in future studies aiming to examine the sex ratio of the lesser horseshoe bat, both in Ireland and in other parts of its range.

3.5 Conclusion

- Sequence data was successfully obtained for the ZFX, ZFY, SRY and DBY genes of *Rhinolophus hipposideros.*
- A real-time PCR sex determination assay for *R. hipposideros* has been designed and validated, comprising an internal control targeting the ZFX gene and two male-specific primers targeting the SRY and DBY genes.

Chapter 4

Assessment of the sex ratio of adult bats in lesser horseshoe bat summer roosts in Ireland

4.1 Introduction

During the course of the 20th Century, the lesser horseshoe bat, *Rhinolophus hipposideros*, suffered a catastrophic population decline in much of its range in Europe and became locally extinct in some areas. As a result, it is a species of particular conservation concern in Europe, with special protection in European wildlife legislation, and special efforts to safeguard its current population and encourage future growth (Dietz et al., 2009).

As one of Ireland's rarest bat species, limited to a narrow range along the western seaboard, the lesser horseshoe bat population has been continuously monitored by the National Parks and Wildlife Service (NPWS) since 1986. This monitoring scheme has been used to provide a national population estimate, which is an important measure of the future viability of this species in Ireland. The most recent population estimate is approximately 14,010 individuals. As discussed in the general introduction, this population estimate is obtained from the mean count data from two consecutive summer monitoring seasons (Roche et al., 2012).

In addition, while the majority of the monitored summer roosts in Ireland are assumed to be maternity roosts dominated by breeding adult females, a significant proportion of adult males is also thought to be present. At present, it is assumed that 25% of adult bats present at monitored summer roosts in Ireland are to be males and the remaining 75% adult females, with the remainder of the adult male population thought to live in small numbers in other unmonitored locations (Roche et al., 2015). However, there is no experimental data on the sex ratio of adult bats at lesser horseshoe bat roosts in Ireland, and the 25%/75% male-female ratio used in population estimation is based on expert opinion. If the ratio of adult male and female bats found in lesser horseshoe bat summer roosts were found to vary significantly from this assumed value, the true population size of this species in Ireland could be substantially higher or lower than previously thought.

In a radio-tracking study of lesser horseshoe bats at a summer maternity roost in southern Wales, Bontadina et al. (2002) found that 24.4% of the adult bats which were trapped were males. However, trapping could not be carried out within the roost itself so as to avoid disturbance to the breeding bats. Thus, bats were trapped a short distance away and less than a third of the total population (90 out of c. 300) was trapped.

Non-invasive genetic sampling offers a means of sampling the entire population of a summer maternity colony while minimising the risk of disturbance to breeding bats. Bat droppings are

a readily collected, high quality source of non-invasive DNA samples (Boston et al., 2012). Genotyping data of sufficient quality to derive population estimates from several lesser horseshoe bat roosts was obtained by Puechmaille and Petit (2007). Recently, Zarzoso-Lacoste et al. (2018) used non-invasive genetic methods to examine the sex ratio of adult lesser horseshoe bats at 19 maternity roosts in northern France. In this study, the authors subjected *R. hipposideros* faecal DNA collected from these roosts to genotyping and sex determination analysis, and found that the overall proportion of males present was 25.8%, but ranged from 0-50%. The mean proportion of males found by this study is very close to the current value used in population estimation for Ireland.

However, a single study from one European country is not a sufficient basis for assumptions about the behaviour of this species across its range. This is particularly the case for lesser horseshoe bat populations in the British Isles, which are further north than any other population of the entire species, and especially Ireland, which sits on the far north-western fringe of the species' range. Unlike the French populations examined by Zarzoso-Lacoste (2018), the Irish lesser horseshoe bat population in some respects inhabits a sub-optimal region for this species, in terms of its cool, wet climate, the relatively small area of suitable foraging habitat resulting from a low percentage of woodland cover, and a limited pool of suitable roost sites, many of which are currently deteriorating to the point that they are no longer useable (Roche et al., 2015). Thus, the proportion of males present in summer maternity roosts in Ireland may be very different to that seen in Zarsozo-Lacoste et al.'s (2017) study due to a limited roost resource. While summer maternity roosts in France were found to contain a low percentage of males overall, in Ireland male lesser horseshoe bats may be forced to occupy summer maternity roosts due to a lack of other available roosting sites, or because the cool climate may require them to occupy the warmer roost sites which are also favoured by females for raising their young.

Therefore, in order to provide accurate data on colony sex ratios to inform the calculation of national censuses of this species it is crucial to examine this question within the Irish context, instead of relying on studies from other countries, as the novel sex typing assays described in Chapter 3 provide the appropriate tool to do this, and the approach of using a real-time PCR assay will also provide the advantage of reduced laboratory workload in comparison to Zarzoso-Lacoste et al.'s (2018) DNA sequencing approach to sex typing of individual animals. The aim of this study was to examine the sex ratio of lesser horseshoe bats at summer roosts in Ireland, and assess how this may affect the national population estimate. This was carried out

using the species identification assay and sex determination assay described in Chapters 2 and 3, respectively, as well as genotyping using a panel of published microsatellite markers.

4.2 Materials and Methods

4.2.1 Site selection

In order to select suitable study sites, the NPWS lesser horseshoe bat summer roost database (currently managed by Bat Conservation Ireland) was accessed. Potential sites were assessed for suitability with Bat Conservation Ireland staff and NPWS wildlife conservation rangers responsible for roost monitoring. Six summer roosts were selected for sampling, based on ease of access to the roosting space and entrances used by bats, geographical spread, and a variety of colony sizes. The sites selected are listed in Table 4.1 and are mapped in Fig. 4.1. Previous monitoring count data is shown in Table 4.2.

Table 4.1: Lesser horseshoe bat roosts in Ireland selected for sex ratio study, from north to south.

Site location	Site code	County	Grid reference (Irish Grid)
Ballykine House, Cong	686	Mayo	M 107 563
Garryland Lodge, Gort	226	Galway	M 412 039
Toonagh House, Ennis	136	Clare	R 308 822
Curragh Chase House, Askeaton	659	Limerick	R 410 490
Courtney's Cottage, Killarney National Park	505	Kerry	V 974 857
Derrycreha House, Glengarriff	193	Cork	V 954 549

Table 4.2: Colony count data 2014-2015 from the NPWS national monitoring scheme for the lesser horseshoe bat for each of the six selected sites in this study.

Site	Count date	Colony count	2014-2015 mean colony count
Ballykine	28/05/2014	178	191
	02/07/2014	203	
	18/06/2015	193	
Garryland	10/06/2014	83	107
	30/06/2015	131	
Toonagh	18/06/2014	30	35
	16/06/2015	40	
Curragh Chase	24/06/2014	82	71
	28/05/2015	60	
Courtney's Cottage	04/06/2014	136	300
	08/07/2014	354	
	30/06/2015	318	
	08/07/2015	390	
Derrycreha	07/07/2014	80	73
	07/07/2015	65	

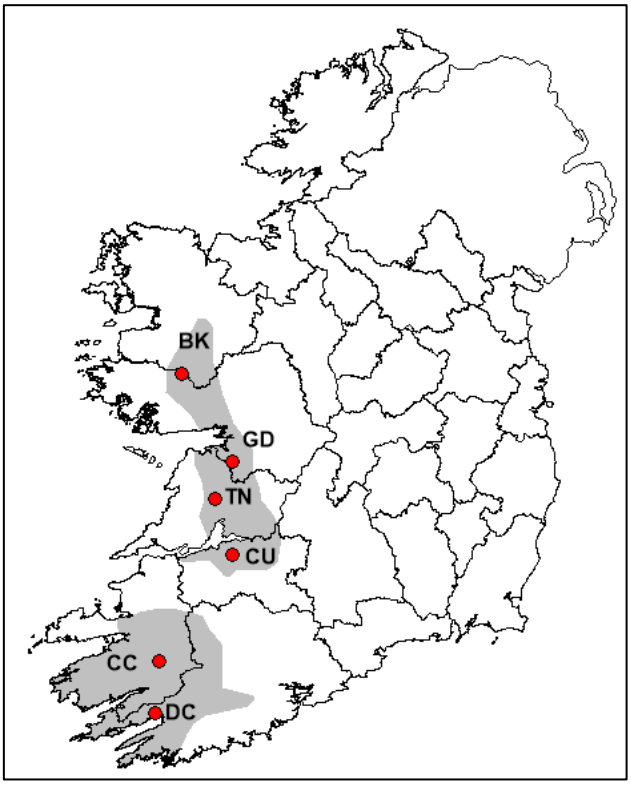

Figure 4.1: Map of roosts selected for study of sex ratio of lesser horseshoe bats. DC- Derrycreha House, Co. Cork; CC- Courtney's Cottage, Co. Kerry; CU- Curragh Chase House, Co. Limerick; TN- Toonagh House, Co. Clare; GD- Garryland Lodge, Co. Galway; BK- Ballykine House, Co. Mayo. Range of lesser horseshoe bat in Ireland highlighted in grey.

4.2.2 Sample collection

The selected bat roosts were visited for sample collection between mid-May and mid-July 2016. This period was selected as the time when adult bats would be present at summer roosts, but before the young bats born that year began to fly, in order to obtain samples from adult bats only. This time also coincided with the period during which summer roost monitoring occurred. Sampling of bat roosts was carried out with the help of local NPWS wildlife conservation rangers, under licence from the NPWS (licence number DER/BAT 2016-29).

Fine mesh plastic netting (Enviromesh®) tied onto $1m^2$ bamboo frames as well as sections of plastic sheeting were used as floor coverings in order to collect bat droppings. Plastic netting was tested for this purpose as it was thought this would allow air circulation to rapidly dry out bat droppings (H. Schofield, pers. comm.). Rapid drying of fresh bat droppings has been shown to improve the quality of DNA subsequently extracted (Boston et al., 2012). Where additional floor covering was required, plastic sheeting was used (empty fertiliser bags were used for this purpose). Floor coverings were left in place to accumulate bat droppings for three to thirteen days between sample collection sessions.

Floor coverings for dropping collection were placed at the entrances to each bat roost. This area of the roost was targeted in order to minimise the risk of sampling bias towards either sex, which could occur if clusters of roosting bats within the roost were targeted for sample collection instead. It was assumed that regardless of where bats of either sex roosted within a particular building, they would all have to leave and return via the same roost entrances (in situations where a limited number of entrances were present).

In addition, the collection of bat droppings at roost entrances was intended to take advantage of the light-sampling behaviour which lesser horseshoe bats undertake prior to emerging from their roosts at dusk, during which time they repeatedly fly just inside the roost entrance. As the bats would spend a substantial amount of time flying in this area of the roost each night and simultaneously depositing droppings, it was surmised that this would be the most suitable area of the roost for collection of a sufficient quantity of droppings.

In a study of brown bears, *Ursus arctos*, in Sweden, Solberg et al. (2006) recommended that non-invasive genetic studies aiming at sampling an entire population obtain between 2.5- 3 times as many samples as the assumed number of animals in the population being studied. In a non-invasive genetic study of lesser horseshoe bat roosts in northern France, Puechmaille and Petit (2007) collected three times as many bat droppings as the number of lesser horseshoe bats

visually counted at each roost. Therefore, this study aimed to collect 2.5-3 times as many droppings as the estimated number of bats present at each site. Estimates for the number of bats present were obtained either from visual counts carried out in daytime while setting up equipment for sample collection, or from dusk emergence counts carried out as part of the national monitoring scheme (provided these occurred while sample collection was in progress).

4.2.3 DNA extraction and species identification

DNA was extracted from bat dropping samples as described in Section 2.2.1.2. DNA extracts were identified to species as described in Section 2.2.1.4, using the RhipCytbF/R primer set.

4.2.4 Microsatellite analysis

Fourteen microsatellite markers were screened for use with *R. hipposideros* DNA samples (Table 4.3). The markers RHA101, RHA105, RHA107, RHA109, RHA7 and RHA8 were initially tested for use as a microsatellite panel, as this was used by Dool et al. (2013) in a large-scale population study of lesser horseshoe bats across Ireland and Europe. Later, the remaining markers (RHC108, RHD102, RHD103, RHD111, RHD113, RHD119, RHD2 and RHD9) were tested as a separate microsatellite panel, following their use by Puechmaille and Petit (2007). Microsatellites were examined for their efficacy in amplifying faecal DNA samples, to reflect the type of non-invasively collected DNA samples which were intended to be used in this survey. All microsatellites were initially amplified in singleplex reactions, which was followed by the testing of multiplex reactions containing several primer sets to increase sample throughput. All microsatellite primers were labelled with a FAM (fluorescein amidite) fluorescent label during singleplex reactions, but fluorescent labels were subsequently altered for multiplex reactions to prevent confusion between different markers during genotype scoring.

PCR reactions for microsatellite genotyping consisted of 5 µl of GoTaq HotStart Green Master Mix (Promega), 5 µM of each primer, 2 µl of faecal DNA and 2 µl of H_2O, to a total volume of 10 µl. In order to maximise efficiency and obtain as many full genotypes as possible, only DNA samples which displayed a Ct value <25 when tested using the RhipCytbF/R primers were selected for this analysis, as samples with a higher Ct value were considered to be likely to be at risk of genotyping errors such as allelic dropout. Each sample was tested in triplicate in order to provide a consensus genotype, due to the risk of genotyping errors. The PCR

conditions consisted of an initial hold at 95°C for five minutes, followed by 50 cycles of 95°C for 30 seconds, 57°C for 90 seconds and 72°C for 30 seconds, with a final extension at 60°C for 30 minutes. Fragment analysis of PCR products was outsourced to Source Bioscience Ltd. Alleles were subsequently scored using Peak Scanner version 2.0 (Applied Biosystems).

Table 4.3: Microsatellite loci tested in this study for amplification with *R. hipposideros*.

Locus	Source	Target species	Primer (5'-3')	Repeat motif	Fluorescent label	Expected size range
RHA101	Struebig et al., 2011	*R. lepidus*	F: GTCAAAGGTTTACCTCCACTCA R: GTTTCATGAAAGAGCCACAGAACATA	$(CA)_{11}$	HEX	131-153
RHA105	Struebig et al., 2011	*R. lepidus*	F: AAGTGCTGGGGACAGAATG R: GTTTGGTTGTTTCGGTGGTCAAT	$(CA)_9$	TAMRA	172-190
RHA107	Struebig et al., 2011	*R. trifoliatus*	F: TCAAGGTCCATCCATGTA R: TGGAAACAATGTAAGTGTGTAC	$(TG)_{16}$	FAM	127-159
RHA109	Dool et al., 2013	*R. hipposideros*	F: AGTGGGACTAAGCCTAACTGAG R: GTTTACGGTGGGACATAAGTAAGAAT		HEX	170-198
RHA7	Struebig et al., 2011	*R. trifoliatus*	F: GCATCTGGCACCCTACTAAGTA R: GTTCTTTTTTTCTACTGCTGCCCTCTAA	$(CA)_{18}$	TAMRA	222-258
RHA8	Struebig et al., 2011	*R. lepidus*	F: ATAGCCTTATTGTTCAGAAGCA R: GTTTATTGGGAGGTCAGAGGAA	$(CA)_{19}$	FAM	137-176
RHC108	Puechmaille et al., 2005	*R. hipposideros*	F: CGAACTGACTCTTCACCAAG R: TTGAGGTCGGAGGGATAG	$(ATTT)_8$	FAM	209-225
RHD102	Puechmaille et al., 2005	*R. hipposideros*	F: TTTCCAGTAGAGCAGATGG R: AAGAACTTTAGAGGGGTTGAT	$(TCTA)_{1+2+1+13}$ $(TCTG)_5$	PET	243-267
RHD103	Puechmaille et al., 2005	*R. hipposideros*	F: CCACTTGGCTTCCTACCT R: CAGAAGGATAGGTTGTTCAATC	$(TCTA)_{1+13+1}$	NED	216-236
RHD111	Puechmaille et al., 2005	*R. hipposideros*	F: ACATGGCACACACACATAC R: CCACCAAAAGGACAAGTAC	$(TAGA)_{5+11}$	NED	280-300
RHD113	Puechmaille et al., 2005	*R. hipposideros*	F: CCTCCTGTCACTCCTTACCC R: CACCGCAAGAAGAAAACATC	$(GATA)_{12+1+2+2}$ $(GACA)_{1+2}$	VIC	208-232
RHD119	Puechmaille et al., 2005	*R. hipposideros*	F: CCTGCTTGCTCTGTCTGTTTA R: CATCCACCATTTCACTGTGTC	$(TCTA)_{2+10}$	PET	144-156
RHD2	Puechmaille et al., 2005	*R. hipposideros*	F: CAAGATGATAAATACGTAGG R: ACAGAGTTAAGAAATACAGG	$(GATA)_{15}$	NED	126-164
RHD9	Puechmaille et al., 2005	*R. hipposideros*	F: GATGGATGGATGGATGGATA R: TCTCCTACTCCTGGTCTACCT	$(TCTA)_{16}$ $(TCCA)_{10}$	VIC	150-174

4.2.5 Microsatellite data analysis

In order to assess the level of genotyping errors, the data obtained was first examined using Micro Checker version 2.2.3 (van Oosterhout et al. 2004), using default settings. This software was used to check the data for possible genotyping errors, including null alleles, allelic dropout and false alleles.

Genalex version 6.503 (Peakall and Smouse, 2006) was used to identify unique genotypes. This programme was also used to assess the number of alleles per locus (A) observed and expected heterozygosity (H_O and H_E), to test for deviations from Hardy-Weinberg equilibrium (HWE), and to estimate the probability of identity (PI) and probability of identity of siblings (PI_{SIBS}).

4.2.6 Capture-Mark-Recapture analysis

CAPWIRE (Miller et al., 2005) was used to obtain population estimates for each roost studied, based on the capture-mark-recapture data for the *R. hipposideros* individuals identified at each site. For each site, numerical data for the number of times each individual bat was "captured" (i.e. the number of times each unique genotype was found) was used as the input data for CAPWIRE. The default parameters were altered to allow for 10,000 bootstraps, 10,000 replicates for the likelihood ratio test to select the most appropriate model (selected from the Even Catchability Model, ECM, or the Two Innate Rates Model, TIRM), and a maximum possible population size of 200 individuals. Additional statistical analysis was carried using Minitab version 17.

4.2.7 Sex typing analysis

All DNA samples which displayed a Ct value <25 when tested using the RhipCytbF/R primers were selected for sex typing analysis, as these were considered to have the highest chance of containing adequate nuclear DNA. Sex typing analysis was carried out using the real-time PCR assay designed in this study as described in Chapter 3, specifically the primer sets RhipZFX3F/R (used as an internal control), RhipSRYhmgF/R and RhipDBY7F/R (both used to identify male individuals). Samples from a single roost (Toonagh) were sex typed using both male-specific primer sets in order to confirm that these consistently identified the same individuals as males, while samples from the remaining roosts were tested using only one male-specific primer set, RhipDBY7F/R. Real-time PCR reactions were carried out as per section 3.2.5.

4.3 Results

4.3.1 Sample collection

Prior to sample collection, the minimum number of bat droppings required for capture-mark-recapture analysis was calculated by estimating the number of bats present at each site. The number of bats present was assessed through a daylight count at small sites where the roosting bats were clearly visible. At larger roosts, where bats could not be seen or were scattered throughout the site during the day, dusk emergence counts by the author, NPWS rangers or BCIreland staff were used to obtain this information. Four of the six roosts (Garryland, Curragh Chase, Courtney's Cottage and Derrycreha) showed a population number for 2016 which was similar to that counted in the previous year (Table 4.4).

Table 4.4: Number of lesser horseshoe bats counted at study sites in 2016.

Site	Count type	Date	Counted by	No. of bats
Ballykine (count 1)	Interior inspection	16/06/2016	J. Higgins, A. Harrington	30
Ballykine (count 2)	Dusk emergence	21/06/2016	J. Higgins	35
Garryland	Dusk emergence	13/06/2016	T. Aughney	110
Toonagh	Dusk emergence	14/06/2016	S. Biggane	57
Curragh Chase	Dusk emergence	31/05/2016	L. Lenihan	80
Courtney's Cottage (count 1)	Dusk emergence	31/05/2016	A Harrington	245
Courtney's Cottage (count 2)	Dusk emergence	07/06/2016	K. Freeman	382
Courtney's Cottage (count 3)	Dusk emergence	07/07/2016	K. Freeman	157
Derrycreha	Interior inspection	18/06/2016	C. Heardman	65

Toonagh showed a proportionally large increase on the previous year, from 40 to 57 bats. This roost had been completely abandoned by the resident bat colony several years previously as a result of a female pine marten (*Martes martes*) entering the roost space and using it as a maternal den (S. Biggane, pers. comm.). However, measures to exclude the pine marten resulted in the bats gradually returning, so the increase in numbers in 2016 may represent the continued recovery of this colony.

Ballykine experienced a major decline in the number of bats visually counted in 2016 compared to the previous year, from 193 to 35. A lower than expected number of bats was noticed when the roost was first inspected during the set-up of sampling equipment (approximately 30), but

it was thought that more could have been present in inaccessible areas of the attic space used by the bats. The extent of the decline in numbers was only noticed when sampling had been ongoing for several days. This was found to have probably resulted from pine marten activity, as one was seen inside the roost building by the author during sample collection.

Although samples were initially intended to be collected exclusively from the roost entrances used by the bats, in practice this was not possible at all of the sites. Roost buildings varied greatly in their accessibility, internal layout and risk of disturbance to bats. The roosts at Garryland, Curragh Chase and Courtney's Cottage proved suitable for the intended strategy of collecting bat dropping samples at the roost entrances. All three sites were managed specifically for their lesser horseshoe bat colonies. These sites contained a summer roosting space consisting of a large, open upper storey or attic with only one or two entrances for the bats, all of which were partially blocked by a wooden partition wall (a "light baffle") to prevent daylight from entering the roost. The "porch" formed by these baffles provided an ideal location for the placement of floor coverings for collection of bat droppings.

In contrast, samples could not be collected exclusively from roost entrances at Ballykine, Toonagh and Derrycreha. Toonagh and Derrycreha were both suboptimal roosts, with relatively small, brightly lit roosting spaces, and with multiple roost entrances in the case of Derrycreha. As the roosting bats were located in a single large cluster at both of these sites, sample collection equipment was placed directly under these. The roost at Ballykine had a complex layout, with a long, narrow roosting space in an attic, and three main entrances used by the bats. Sampling equipment was placed at all three entrances, and underneath a roosting cluster of bats located next to one of the entrances.

During each sampling session, bat droppings which had accumulated on plastic netting and sheets were collected in plastic tubs and either frozen at -20°C or DNA extracted within 24 hours. DNA was extracted from all of the bat droppings collected from Garryland and Courtney's Cottage to provide enough samples for capture-mark-recapture analysis. At the other four sites, several hundred droppings were collected, far in excess of what was required. A subset of the droppings collected from each of these sites was used for DNA sample extraction (Table 4.5).

Table 4.5: Sampling dates and number of DNA samples extracted from each sampled roost.

Site	Sampling dates	No. of samples collected	No. DNA samples extracted	Estimated no. of bats present
Ballykine	15/06/16- 28/06/16	~300	120	35
Garryland	07/06/16- 28/06/16	298	298	110
Toonagh	08/06/16- 14/06/16	~400	150	57
Curragh Chase	08/07/16- 14/07/16	~300	200	80
Courtney's Cottage	26/05/16- 01/06/17	393	393	245
Derrycreha	18/06/16- 24/06/16	~400	180	65
Total			1341	592

4.3.2 Species identification

Real-time PCR species identification testing identified 1,264 of the 1,341 DNA samples (94%) as having originated from *R. hipposideros* (i.e. samples with a Ct value < 30). The mean Ct value of samples identified as *R. hipposideros* from the six sites ranged from 18.44 to 20.42 (Table 4.6). The Ct value obtained for each DNA sample was used to screen samples for further analysis using microsatellite markers. Samples with a Ct value < 25 were selected for amplification with the panel of microsatellites, while samples with a Ct > 25 were excluded from further analysis as they were thought unlikely to contain sufficient nuclear DNA for successful microsatellite genotyping or sex typing. In total, 1237 samples (92% of the total) were deemed to be suitable for microsatellite genotyping and sex typing analysis.

Table 4.6: Summary of real-time PCR results for species identification of *R. hipposideros*.

Site	No. DNA samples	No. *R. hipposideros* samples	*R. hipposideros* Samples Ct < 25	*R. hipposideros* mean Ct
Ballykine	120	116	116	18.47 ± 1.25
Garryland	298	286	276	20.42 ± 2.47
Toonagh	150	148	148	18.66 ± 1.26
Curragh Chase	200	174	170	20.01 ± 1.57
Courtney's Cottage	393	360	356	19.11 ± 2.33
Derrycreha	180	173	171	18.37 ± 1.72
Total	1341	1257	1237	

Significant differences (Kruskal-Wallis test, H = 178.22, 5 df; P < 0.01) were observed between the Ct values for the DNA samples collected from the six roosts. Significant differences were found between Ct values of DNA samples from Garryland and Curragh Chase (with the highest mean Ct values); Courtney's Cottage; Ballykine and Toonagh; and Derrycreha, with the lowest mean Ct values (Fig. 4.2)

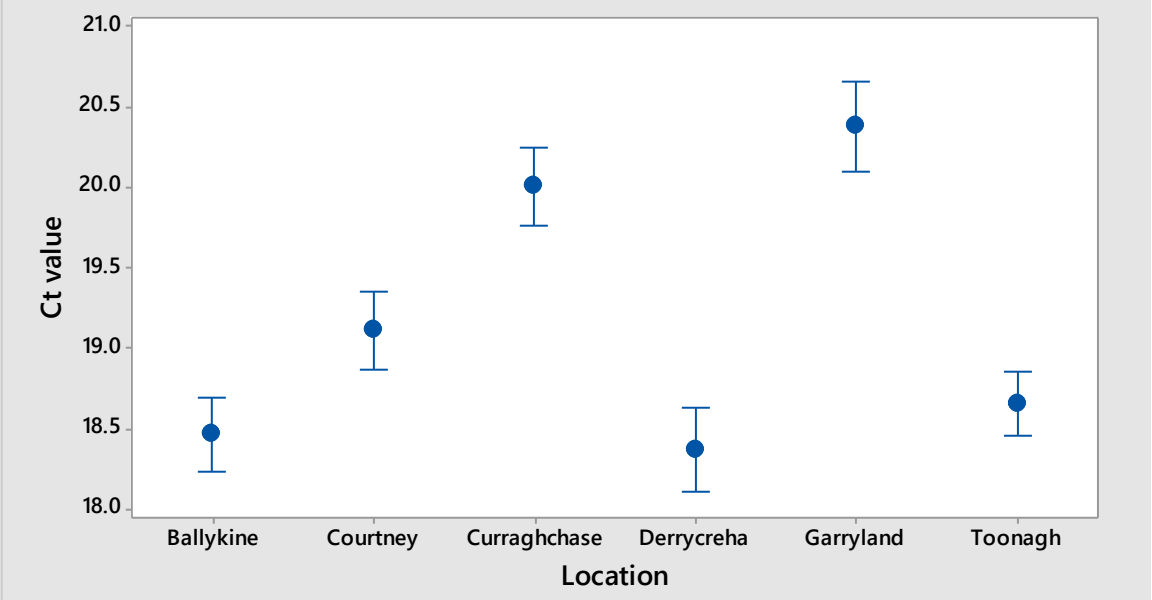

Figure 4.2: Interval plot showing the mean species assay Ct values of DNA samples from the six surveyed *R. hipposideros* roosts. 95% confidence intervals indicated by error bars.

The species assay Ct values for the DNA samples obtained from Curragh Chase were also used to examine the efficacy of the plastic netting ground covering in drying out bat droppings and thus preserving the quality of the DNA they contained, in comparison to plastic sheeting. Bat droppings which were collected at the main entrance used by the bats at this roost with both plastic netting and sections of plastic sheets were used for this analysis, as the ground covering from which each sample originated had been noted during their collection. In total, 92 of the extracted DNA samples used in the analysis had been collected on plastic netting, and 70 had been collected on plastic sheets, all of which had been confirmed as having originated from *R. hipposideros*. Although the mean Ct values obtained for DNA samples collected from netting and plastic sheets were very similar (20.11 ± 1.48 and 19.89 ± 1.68, respectively), the Ct values for the samples collected using the plastic sheets were found to be significantly lower (Mann-Whitney U test (P < 0.05).

4.3.3 Microsatellite optimisation

Fourteen microsatellite markers were investigated, divided into two separate panels. The first of these consisted of six markers (RHA101, RHA105, RHA107, RHA109, RHA7 and RHA8). These loci were used as part of a microsatellite panel by Dool et al. (2013) to amplify *R. hipposideros* tissue DNA. However, RHA101, RHA105, RHA107 and RHA5 either failed to amplify or amplified poorly with the faecal DNA samples used in this study, so this microsatellite panel was not used further.

The second microsatellite panel consisted of the remaining eight markers (RHC108, RHD102, RHD103, RHD111, RHD113, RHD119, RHD2 AND RHD9), as used by Puechmaille et al. (2005) and Puechmaille and Petit (2007) to amplify faecal DNA. All of these markers amplified the faecal DNA samples used in this study, with the exception of RHD9 which failed to amplify the majority of samples. As a result RHD9 was not used further in this study.

Two microsatellites were redesigned in order to reduce the overlap with other markers of similar size, thus making scoring of microsatellites easier in a multiplex reaction. RHC108 and RHD103 were redesigned to reduce the overlap in expected allele size seen in both of these microsatellites and RHD113, all of which fell in the range of 208-236. The sequences for both microsatellites were obtained from Genbank, and primers redesigned using Primer Express v2.0 (Applied Biosystems). The reverse primer targeting RHC108 was redesigned to increase the expected allele size by 20 base pairs. The forward primer targeting RHD103 was redesigned to decrease the expected allele length by 37 bp (Fig. 4.3).

>gi|70986605|gb|DQ102689.1| Rhinolophus hipposideros clone RHC108 microsatellite sequence

CTTAAACAAACGAACTGACTCTTCACCAAGTCTGTCTTATTCTTAGAAATTCTTATACGAAATCCATGGTGAGG

CCCATTTTTTATTATTATTTATTTATTTATTTATTTATTTATTTATTAGTTTCAGGTGTACAAGACAAGGTAA

TACTTAGACGTTCATCATTTATATCCCTCACACTGTGAACCCCCCTCCCCCCATCCACTATCCCTCCGACCTCAAA

CTGAACCATCACATTATATATATAAAATTATAGTTGG

>gi|70986763|gb|DQ102693.1| Rhinolophus hipposideros clone RHD103 microsatellite sequence

CAAGAGGCTCAGTCTATAGGCCACTTGGCTTCCTACCTCATCCTCACTCTTAAGCAGACATTGAGTAAAA

TGTGACTGAAACGAATTAAATCAATTATCTATCATCTATCTATCTATCTATCTATCTATCTATCTATCTA

TCTATCTATCTATCTATCATCTATCCATCTCAAATATACTCCAAATGGAAGTATATTGTACAGGAAGAAG

ATTANAGGAAGATGGCAGATTGAACAACCTATCCTTCTGACATTAAGTATATATATAATTTTTTTAAATG

Figure 4.3: RHC108 microsatellite sequence showing the position of the original forward and reverse primers (highlighted in grey) from Puechmaille et al. (2005). The reverse primer was redesigned for this study (in green, underlined) to increase the length of the amplicon by 20 bp. (Below) RHD103 microsatellite sequence showing the position of original forward and reverse primers from Puechmaille et al. (2005), and the redesigned forward primer to decrease the amplicon length by 37 bp.

4.3.4 Multiplex PCR

The microsatellite panel selected for use in this study had been previously used in a single multiplex reaction by Puechmaille et al. (2005) and Puechmaille and Petit (2007). However, when multiplex reactions were carried out using equal concentrations of each primer set in this study, problems were encountered with the amplification of certain markers. In particular, RHD103 completely failed to amplify, and RHD102 and RHD119 displayed peaks so small as to make scoring of genotypes very difficult. This contrasted with singleplex reactions, where all of the microsatellite markers successfully amplified, with much larger peaks than seen in the multiplex reactions.

Instead, two separate multiplexes were developed, one containing RHC108b, RHD103b and RHD2, and the other containing RHD102, RHD111, RHD113 and RHD119, with primer

concentrations ranging from 0.5 to 1.0 µM (Table 4.7). By examining the peak heights of individual microsatellite loci during scoring, primer concentrations were varied to allow equal amplification of products. After amplification, multiplex products for each sample were combined before fragment analysis (Fig. 4.4).

Table 4.7: Final microsatellite panel used in this study.

Locus	Source	Primer (5'-3')	Multiplex	Panel conc. (µM)	Label	Observed size range
RHC108b	This study	F: CGAACTGACTCTTCACCAAG R: ATATAATGTGATGGTTCAGTTTGAG	1	0.75	FAM	232-236
RHD102	Puechmaille et al., 2008	F: TTTCCAGTAGAGCAGATGG R: AAGAACTTTAGAGGGGTTGAT	2	1.0	FAM	243-267
RHD103b	This study	F: ACATTGAGTAAAATGTGACTGAAAC R: CAGAAGGATAGGTTGTTCAATC	1	0.75	NED	176-208
RHD111	Puechmaille et al., 2008	F: ACATGGCACACACACATAC R: CCACCAAAAGGACAAGTAC	2	0.75	FAM	274-302
RHD113	Puechmaille et al., 2008	F: CCTCCTGTCACTCCTTACCC R: CACCGCAAGAAGAAAACATC	2	0.5	VIC	207-231
RHD119	Puechmaille et al., 2008	F: CCTGCTTGCTCTGTCTGTTTA R: CATCCACCATTTCACTGTGTC	2	1.0	PET	143-163
RHD2	Puechmaille et al., 2008	F: CAAGATGATAAATACGTAGG R: ACAGAGTTAAGAAATACAGG	1	1.0	FAM	122-170

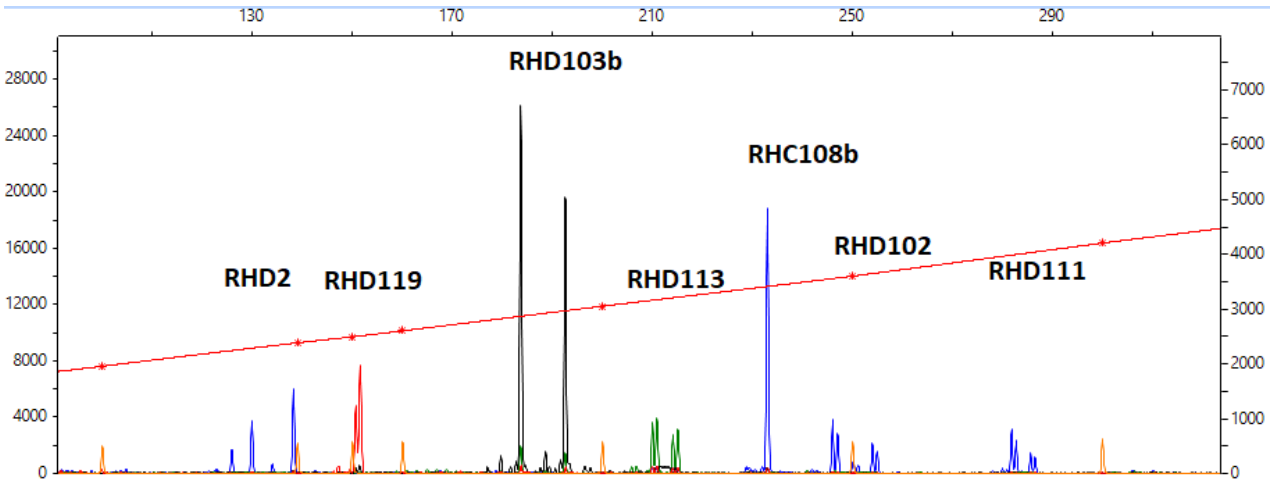

Figure 4.4: Example of multiplex genotype for R. hipposideros, with microsatellite marker peaks labelled.

4.3.5 Microsatellite genotyping of field samples

Due to constraints of time and cost, three out of the six sites included in this study (Ballykine, Toonagh and Derrycreha) were selected for genotyping of all DNA samples, in order to identify all individual bats present.

In total, 435 faecal DNA samples from the three roosts were subjected to microsatellite genotyping. Consensus genotypes for each sample were deduced from triplicate results. Where ambiguous results were obtained, alleles which occurred in two or more replicates were accepted. Samples for which a consensus genotype could not be obtained were discarded, and only genotypes where all seven markers were scored were used for analysis.

Full genotypes were successfully obtained from 396 samples (91%), from which 180 unique genotypes were identified (Table 4.8). The number of replicated genotypes varied from one to ten recaptures. The unique genotypes obtained are listed in Appendix 6.

Table 4.8: Number of *R. hipposideros* individuals identified per roost via microsatellite genotyping.

Roost	**No. samples genotyped**	**No. genotypes obtained**	**No. individuals identified**
Ballykine	116	114	63
Toonagh	148	127	53
Derrycreha	171	155	64

Analysis with Micro Checker found no evidence of scoring errors due to stuttering, large allele dropout, or null alleles. The number of alleles identified ranged from two at RHC108b to twelve at RHD2, averaging at seven alleles per locus. The overall observed heterozygosity ranged from 0.178 to 0.828 (in RHC108b and RHD113, respectively), and averaged 0.622. Expected heterozygosity ranged from 0.171 to 0.878 (in RHC108b and RHD2), averaging 0.650. For all roosts, two loci, RHD119 and RHD2, were found to deviate significantly from Hardy-Weinberg equilibrium ($P < 0.001$). However, when each roost was analysed separately only one instance of deviation from Hardy-Weinberg equilibrium was found, in RHD2 at Ballykine. RHC108b was found to be monomorphic at Ballykine. Summary statistics are provided in table 4.9.

Table 4.9: Descriptive statistics for the lesser horseshoe bats sampled, both for the entire sampled population, and according to each roost. Number of samples amplified per locus (N), number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and Hardy-Weinberg expectations (HWE). * denotes deviation from Hardy-Weinberg equilibrium.

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2	Average
All sites								
N	180	180	180	180	180	180	180	180
A	2	6	7	8	7	7	12	7
H_O	0.178	0.389	0.689	0.744	0.828	0.772	0.756	0.622
H_E	0.171	0.445	0.686	0.794	0.774	0.801	0.878	0.650
HWE	0.598	0.000*	0.220	0.078	0.725	0.623	0.000*	0.321
Ballykine								
N	63	63	63	63	63	63	63	63
A	1	2	4	5	6	7	10	5
H_O	0.000	0.524	0.635	0.619	0.810	0.810	0.778	0.696
H_E	0.000	0.495	0.603	0.629	0.721	0.810	0.815	0.679
HWE	N/A	0.650	0.837	0.775	0.103	0.393	0.035*	0.466
Toonagh								
N	53	53	53	53	53	53	53	53
A	2	6	6	8	5	6	12	6.4
H_O	0.358	0.377	0.698	0.906	0.830	0.774	0.849	0.685
H_E	0.318	0.425	0.681	0.822	0.720	0.744	0.821	0.647
HWE	0.350	0.113	0.359	0.870	0.777	0.981	0.784	0.605
Derrycreha								
N	64	64	64	64	64	64	64	64
A	2	4	5	7	7	6	7	5.4
H_O	0.203	0.266	0.734	0.734	0.844	0.734	0.656	0.596
H_E	0.182	0.251	0.728	0.773	0.796	0.771	0.617	0.588
HWE	0.366	0.960	0.964	0.436	0.890	0.857	0.892	0.766

The overall probability of identity (PI) for all sites was 4.0×10^{-7}, while the overall probability of identity for siblings (PI_{SIB}), a more conservative measure, was 3.8×10^{-3} (Table 4.10). The observed values for PI and PI_{SIB} are comparable to those obtained by similar non-invasive studies of *R. hipposideros* (Puechmaille et al., 2005; Puechmaille and Petit, 2007; Zarzoso-Lacoste et al., 2018), indicating that the panel of seven microsatellite primers was sufficient to distinguish between individuals. A curve for the PI and PI_{SIB} according to the cumulative number of markers used is shown in fig. 4.5.

Table 4.10: Observed PI and PI_{SIB} values for *R. hipposideros* roosts at Ballykine, Toonagh and Derrycreha, and overall values for all sampled individuals.

	Ballykine	Toonagh	Derrycreha	Overall
PI	7.5×10^{-6}	1.2×10^{-6}	4.7×10^{-6}	4.0×10^{-7}
PI_{SIB}	7.9×10^{-3}	4.3×10^{-3}	7.1×10^{-3}	3.8×10^{-3}

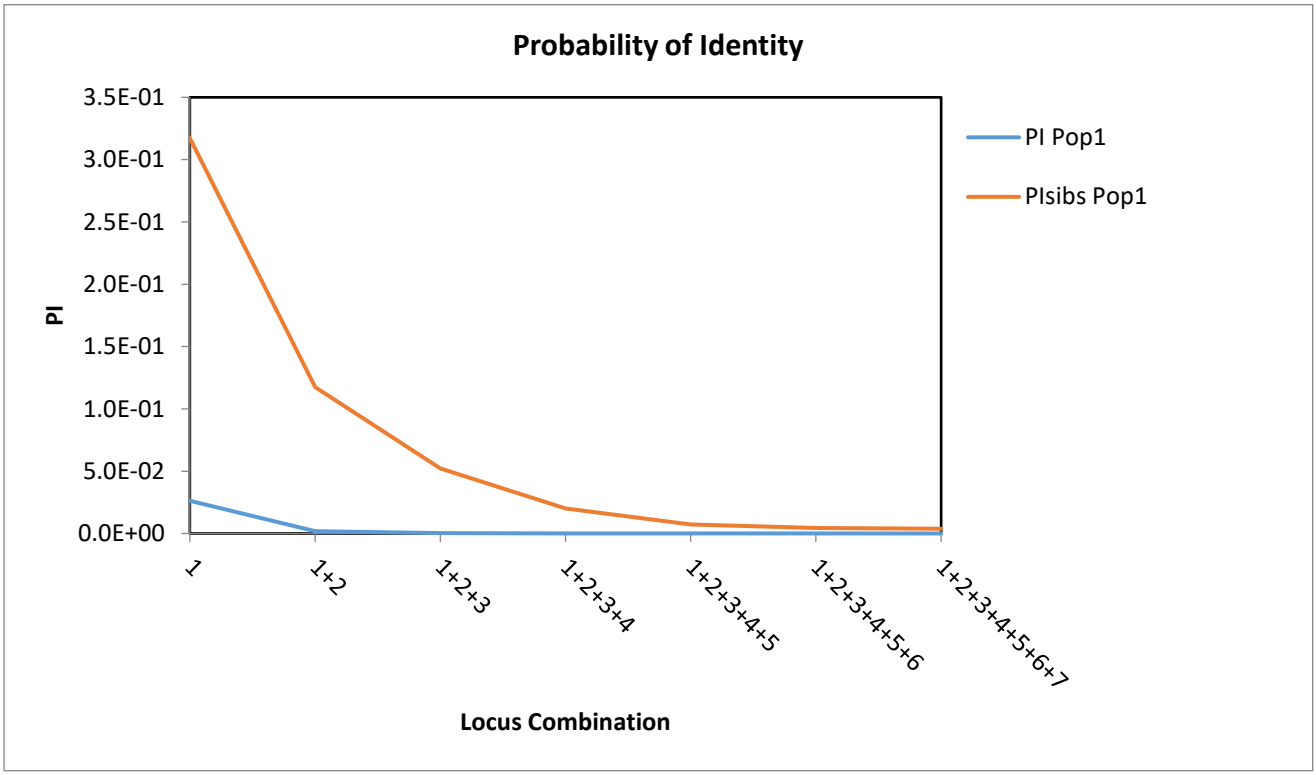

Figure 4.5: PI and PI_{SIB} curves for the sampled *R. hipposideros* population at Ballykine, Toonagh and Derrycreha. Locus combination order: RHD2, RHD103b, RHD102, RHD113, RHD111, RHD119, RHC108b.

4.3.6 Capture-Mark-Recapture analysis

The 180 unique genotypes obtained were subjected to CMR analysis using CAPWIRE. The largest number of recaptures per genotype was ten, with the mean number of recaptures per site ranging from 1.81 to 2.44 (Table 4.11). CAPWIRE gave population estimates for each colony based on the Equal Catchability Model (ECM) and the Two Innate Rates Model (TIRM). Population estimates are shown in Table 4.12 and Fig. 4.6. Population estimates based on ECM were lower than those based on TIRM, and more closely matched visual counts and the number of genotypes obtained.

Table 4.11: Capture frequencies of 178 individual *R. hipposideros* identified at Ballykine, Toonagh and Derrycreha colonies.

Number of times captured	Number of identities	Number of identities	Number of identities
	Ballykine	Toonagh	Derrycreha
1	40	21	19
2	13	13	22
3	3	9	10
4	4	5	5
5	1	2	4
6	0	0	1
7	0	2	1
8	1	0	1
9	0	1	0
10	1	0	0
Total no. of captures	114	127	155
Mean no. of recaptures	1.81	2.40	2.44

Table 4.12: CAPWIRE population estimates for lesser horseshoe bat colonies at Ballykine, Toonagh and Derrycreha, based on the ECM and TIRM models, with 95% confidence intervals (CI) also shown.

Colony	Visual count	No. of genotypes	Population estimate-ECM (95% CI)	Population estimate-TIRM (95% CI)
Ballykine	35	63	85 (71-102)	129 (84-149)
Toonagh	57	53	60 (54-66)	75 (62-93)
Derrycreha	65	64	72 (64-78)	85 (75-106)

4.3.7 Sex typing of field samples

Sex typing analysis was carried out on all samples from Ballykine, Toonagh and Derrycreha for which a full genotype was obtained. In total, 396 samples from these roosts were subjected to sex typing analysis. For the roosts at Curragh Chase, Garryland and Courtney's Cottage, a subset of 150 samples from each roost were subjected to sex typing analysis in order to estimate the sex ratio of bats present at these sites, bringing the total number of sex typed samples to 846.

All of the DNA samples obtained from Toonagh (n = 127) were subjected to sex typing using both of the male-specific real-time PCR primer sets described in Chapter 3 (RhipSRYhmgF/R

and RhipDBY7F/R). Both of the primer sets consistently identified the same samples as having originated from male individuals. DNA samples from the other five roosts were subsequently sex typed using a single male-specific primer set (RhipDBY7F/R).

The proportion of samples from each roost which were found to have originated from male bats was found to range widely, from 14.2% at Curragh Chase to 74.3% at Courtney's Cottage (Table 4.13). 95% confidence intervals were calculated using the formula for the standard error of a sample proportion shown in equation 1, where *e* is the 95% confidence interval, $\hat{p}$ is the sample proportion (i.e. the observed percentage of males), *z* is a constant (in this case 1.96 for a 95% confidence interval) and *n* is the sample number (i.e. the number of faecal DNA samples tested).

$$e = \pm z \sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$$

Eqn. 1

Table 4.13: Summary of sex typing data for DNA samples from the six surveyed *R. hipposideros* colonies.

Site	No. sexed DNA samples	No. male samples	No. female samples	% male samples (95% CI)
Ballykine	114	46	68	40.4 (±9.0)
Toonagh	127	39	88	30.7 (±8.0)
Derrycreha	155	56	99	36.1 (±7.6)
Curragh Chase	148	21	127	14.2 (±5.6)
Garryland	149	86	63	57.7 (±7.9)
Courtney's Cottage	144	107	37	74.3 (±7.1)

The sex typing data and genotyping data from Ballykine, Toonagh and Derrycreha were subsequently combined to assign the sex of each identified individual and thus obtain a more accurate sex ratio for these sites, removing the error in the sex ratio associated with multiple recaptures of individuals. Of the 180 individuals identified at the three roosts, all of the samples from 179 of the individuals gave consistent sex typing results. Only one sample produced an inconsistent result, which originated from an individual from Toonagh. Of the nine samples obtained from this animal, eight were identified as male, and one as female, even after several re-tests. This individual was included in the sex ratio analysis, as the single inconsistent result was ascribed to probable contamination by DNA from a female individual.

When sex typing data was analysed according to the individual animals identified, the percentage of males present ranged from 26.4% at Toonagh to 42.8% at Ballykine (Table 4.14). The sex ratio of individuals from each roost was found to lie within the 95% confidence intervals shown in Table 4.13.

Table 4.14: Summary of numbers of male and female *R. hipposideros* individuals identified at Ballykine, Toonagh and Derrycreha, and percentage of male individuals at each roost, combining sex typing data and genotyping data.

Site	No. individuals sex typed	No. male individuals	No. female individuals	% male individuals
Ballykine	63	27	36	42.8
Toonagh	53	14	39	26.4
Derrycreha	64	23	41	35.9

4.3.8 Application of results to national population estimation

The sex ratio data obtained was applied to the formula which is currently used to estimate the Irish lesser horseshoe bat population, to examine the effect on the most recent estimate. For each of the six roosts, the number of males present was calculated based on the percentage of males detected, and the highest colony count nearest to the period of sampling (either from visual counts or number of individuals genotyped) (Table 4.15). The estimated total number of males present at the six roosts ranged from 292-352, making up 47.1-56.8% of the total number of bats counted.

Table 4.15: Estimation of the number of males present at the six lesser horseshoe bat roosts surveyed.

Site	No. of individuals	% male	Estimated no. of males
Ballykine	63	42.8	27
Toonagh	57	26.4	15
Derrycreha	65	32.9	23
Curragh Chase	80	8.6-19.8	7-16
Garryland	110	49.8-65.6	55-72
Courtney's Cottage	245	67.2-81.4	165-199
Total	620		292-352

The most recent population estimate for the lesser horseshoe bat in Ireland was obtained in 2012, using mean count data from 2010 and 2011 (Roche et al., 2012). In addition, it was assumed that 25% of the bats counted were males, that the entire female population was counted, and that the entire population consists of 1:1 ratio of males to females. Using the data and assumptions, the population estimate was calculated as follows:

Mean count data, 2010-2011		Proportion of female bats		1:1 overall sex ratio		Population estimate
9340	x	0.75	x	2	=	14010

An updated population estimate based on the new data obtained in this study was calculated by adjusting the second figure in the formula above (the proportion of female bats). Based on an estimated proportion of adult male bats ranging from 47.1-56.8%, the updated population estimate for lesser horseshoe bats in Ireland was 8,070-9,882 individuals. As the lower population estimate was less than the actual mean count data, this estimate should be adjusted to 9,340-9,882 individuals, which is 29.5-33.3% lower than the previous population estimate calculated by Roche et al. (2012).

For future survey work to examine the sex ratio of lesser horseshoe bats, the number of DNA samples to be tested to provide a sex ratio estimate within a given margin of error can be calculated by manipulating the formula for the standard error of a sample proportion (equation 1). In order to calculate the number of samples required, *n*, the formula in equation 2 can be used:

$$n = \frac{z^2 \hat{p}(1-\hat{p})}{e^2}$$

Eqn. 2

The maximum number of samples required within for a particular margin of error can be calculated by taking $\hat{p}$ to be 0.5 (i.e. a sex ratio of 50%), as the standard error is largest for this proportion. As an example, the maximum number of samples required to estimate the sex ratio of bats at a roost to a 95% confidence interval of 7% (thus $e = 0.007$ and $z = 1.96$) would require 196 DNA samples to be sex typed.

4.4 Discussion

4.4.1 Sample collection and DNA quality

The collection of droppings from *R. hipposideros* roosts proved to be a successful method of obtaining high quality non-invasive DNA samples of this species, having already been used in previous studies (Puechmaille and Petit, 2007; Zarzoso-Lacoste et al., 2018). However, while these studies only collected droppings from beneath large clusters of bats within their roost, this study successfully collected samples both in this way and from the roost entrances where the bats performed their light sampling behaviour at dusk. While sample collection at the roost entrances was the preferred method in this study, it was not possible to do so at two roosts (Toonagh and Derrycreha) due to an excessive number of roost entrances or risk of excessive disturbance to the bats present. Equally, it would not have been possible or practical to collect droppings solely from under aggregations of bats at other sites, either because they were roosting in an inaccessible attic space (Courtney's Cottage) or they were scattered across a large roost (Curragh Chase and Garryland). The variable layouts of the different roosts surveyed required both methods to be used.

The comparison of the two sample collection materials used (plastic netting and plastic sheets) at Curragh Chase showed the netting did have not the anticipated effect of improving DNA quality in the samples collected through rapid drying, and in fact the plastic sheeting produced DNA samples of significantly higher quality (as measured by real-time PCR species assay Ct values). However, both materials produced high quality DNA samples, and previous studies which used newspaper to collect *R. hipposideros* droppings have also reported that this resulted in high quality DNA samples (Puechmaille and Petit, 2007; Zarzoso-Lacoste et al., 2018).

The square frames of plastic netting were time-consuming to build and in some situations were difficult to deploy in confined spaces because of their rigid shape, but they had the advantage that they could be placed into position very quickly and silently, minimising the disturbance caused to the resident bats. In contrast, the plastic sheets were easily obtained and could be used to cover a large area of roost space, but they took time to unfold and put into position and tended to be very noisy (including at ultrasound frequencies), which could potentially cause disturbance to the bats. Using both collection materials allowed for enough floor space to be covered to collect a large number of bat droppings (especially when several roosts were being sampled simultaneously), and also allowed for flexibility in different roost situations.

Another factor which affected the quality of the DNA samples collected was the site of origin, with the mean Ct values of samples from Curragh Chase, Garryland and Courtney's Cottage being significantly higher than those from Ballykine, Toonagh and Derrycreha. As has been noted above, the roost spaces in the former three sites are relatively large and have been extensively modified for the benefit of the resident *R. hipposideros* colonies, including the placement of light baffles at the one or two entrances at each site. In contrast, the latter three sites contain relatively small roosting spaces and have been less modified for their *R. hipposideros* colonies, with no light baffles inserted. It is possible that the presence or absence of these light baffles has resulted in differing internal conditions within each roost, as the baffles may impede air flow through roosts where they are installed and thus increase the air humidity within. This would affect the drying out of bat droppings being collected, which has been shown to affect their DNA quality (Puechmaille et al., 2007). However, the humidity level in each roost was not measured. Alternatively, differing weather conditions or variation in the diet of the bats at each site, or some other factor, may account for the differences observed.

4.4.2 Microsatellite genotyping and population estimation

Screening of the DNA samples according to the Ct value obtained from the species identification assay allowed the highest quality samples to be preferentially subjected to microsatellite genotyping. Of the 435 samples analysed, 396 produced full genotypes (91%), and when the full genotypes were examined with Microchecker, no evidence of errors including allelic dropout or false alleles was found. This illustrated the success of the approach taken in this study, following that used by a number of similar studies (O'Neill et al., 2013; Sheehy et al., 2014; O'Meara et al., 2018) in using real-time PCR to select only high-quality DNA samples for genotyping in an effort to maximise genotyping success and avoid issues with genotyping errors (Beja-Pereira et al., 2009). Puechmaille and Petit (2007) demonstrated that *R. hipposideros* faecal DNA samples yielded high quality genotyping results, but did encounter low levels of genotyping errors including allelic dropout and false alleles. However, wheareas Puechmaille and Petit (2007) attempted genotyping of all faecal DNA samples collected, this study took a more selective approach, by using only the highest quality DNA samples as measured by real-time PCR species assay Ct values, with an upper cut-off of Ct 25. In addition, only full genotypes were used to attempt to identify individual animals, unlike Puechmaille and Petit's (2007) use of some partial genotypes, and this would also have helped

to ensure only the very highest quality samples were used and helped to eliminate instances of genotyping errors.

The cumulative PI_{SIB} value (0.0038) for the full microsatellite panel of seven loci was below the level of 0.05 recommended by Schwartz and Monfort (2008) for adequate discrimination of individuals, and in fact this level was reached with a cumulative PI_{SIB} for only five markers (0.033). This indicates that the full panel of seven loci had sufficient power to distinguish between all of the individual bats encountered in the sampled roosts.

The average observed (H_O) and expected (H_E) heterozygosity were similar for each of the three roosts sampled, with H_O ranging from 0.596 at Derrycreha to 0.696 at Ballykine. No deviation from HWE was observed at any locus for Derrycreha and Toonagh. At Ballykine, a significant deviation from HWE was seen at RHD2, and RHC108b was found to be monomorphic. Both of these observations may be due to several factors, including inbreeding, a founder effect (low genetic diversity caused by a very small initial colonising population) or a population bottleneck (whereby a population's size falls drastically, deacreasing its genetic diversity) (Rowe et al., 2017), as this roost is located in the far north of the lesser horseshoe bat's range in Ireland.

The population estimates obtained by using CAPWIRE varied depending on the model used, with TIRM giving higher estimates than ECM, and the ECM estimates being closer to the number of bats visually counted and the number of genotypes obtained. Puechmaille et al. (2007) carried out a similar study, comparing population estimates of lesser horseshoe bat colonies from visual counts with those based on non-invasive genetic sampling with CAPWIRE. This study found that TIRM gave higher population estimates with a wider 95% confidence interval than ECM, and ECM gave estimates which were much closer to the number of genotypes counted and the visual counts. This was similar to the pattern seen in this study, which suggests that the ECM population estimates are more appropriate in this case and probably more closely reflect the true situation.

At Derrycreha and Toonagh, the number of recaptures per individual was 2.44 and 2.40 respectively, which is within the 2 to 2.5 range recommended by the CAPWIRE programme to obtain a population estimate within approximately 10% to 15% of the true population size. The ECM population estimates for both sites were higher than the visual counts and the number of genotypes, at 72 (95% CI 64-78) for Derrycreha and 60 (95% CI 54-66) for Toonagh. However, the visual counts for both sites and the number of genotypes obtained from

Derrycreha were within the ECM 95% confidence intervals, and the number of genotypes from Toonagh was just under the ECM 95% CI. This indicates that the majority of individuals from both sites were sampled in this study.

At Ballykine, the number of unique genotypes identified was nearly twice the number of bats observed during a dusk emergence count (63 and 35, respectively), both of which represented a large decline in the population of 193 bats counted the previous summer. The number of recaptures per individual was 1.81, below the recommended level for obtaining an accurate population estimate with CAPWIRE. The ECM population estimate produced by CAPWIRE was 85 (95% CI 71-102), higher than either of the other measures of population size, but still lower than the population level seen in previous years. By this measure, 76.5% of the estimated population had been genetically sampled.

The widely varying population estimates for this colony probably reflect the serious disturbance that it was undergoing, which was unfortunately only fully appreciated once sampling was already in progress. This was most likely caused by the activity of a pine marten within the roost building which was noticed on the day that samples were collected at this site, as this species is known to predate on bats within their roosts in other parts of Europe (Power, 2015) and has been noted to have caused similar disturbance at other lesser horseshoe bat roosts in Ireland (K. McAney, and S. Biggane, pers. comm.). The discrepancy in the three population estimates may indicate that the number of bats was actively falling while sample collection was in progress. Alternatively, the resident bats may have moved to another roost nearby and may have returned periodically to the surveyed roost in small numbers, leading to the number of bats that were visually counted being lower than the number of recorded genotypes.

4.4.3 Sex typing

As with the microsatellite genotyping analysis, the Ct values from the species identification assay were used as a screening method to select the highest quality samples for sex typing analysis. For Ballykine, Toonagh and Derrycreha, only those samples which produced full genotypes were subjected to sex typing analysis. Of these, all 396 (100%) were successfully sex typed. For Curragh Chase, Garryland and Courtney's Cottage, of the 450 samples tested, 441 samples (98%) were successfully sex typed. Screening the DNA samples based on their Ct value from the species identification assay was primarily used to select samples with the best chance of successfully being sex typed, but the screening process was also necessary because

there was a certain amount of non-specific amplification seen in samples with low quantities of target DNA when the sex typing assays were used (Tables 3.7 and 3.12). Therefore, by being selective with the sampled subjected to sex typing, false positives in the sex typing analysis of the samples attributable to non-specific amplification were avoided.

A concern with using this method is that it could potentially bias sex ratio estimates based on selectively analysing only samples containing high quality DNA, if there were any sex-based differences such as hormone content or dietary differences which could contribute to faecal samples from one sex containing significantly higher quality DNA than the other. However, this issue can be discounted for two reasons. Firstly, neither males nor females were found to be predominant in all of the colonies, with the proportion of males ranging from 14% to 74%, whereas a bias towards one of the sexes in all of the colonies could have indicated that samples from that sex were being inadvertently selected during DNA analysis. Secondly, when sex typed samples were ranked according to their species identification assay Ct value, no pattern was seen whereby samples of higher quality were found to be disproportionately from either males or females.

When the sex typing and genotyping data were combined to assign the sex of individual animals, the sex typing results for all recaptured individuals were consistent, with the exception of a single individual from Toonagh. As noted in section 4.3.6, this individual was recaptured nine times. Of these, eight samples were clearly identified as male, while a single sample gave an anomalous female result, even after being retested. This inconsistency may have arisen through contamination of the single sample which gave an anomalous result, where a dropping from a male individual may have been covered in urine from a female individual. Another possibility is that this may have resulted from the misidentification of two individuals of different sexes as one individual, based on the genotyping data. While this is not ideal, the possibility of misidentifying individuals when using microsatellites can never be fully ruled out, although using a large number of microsatellites makes the probability of this occurring extremely small. The probability of identity of siblings, PI_{SIB}, is a conservative measurement used to estimate the probability of just this type of occurrence, calculated based on the particular panel of microsatellites used. As has been noted in section 4.4.2, the overall PI_{SIB} calculated for this study (3.8×10^{-3}) was below the accepted level of 0.05 recommended by Schwartz and Monfort (2008) for adequate discrimination of individuals, and was in the same range as that seen in several similar studies of *R. hipposideros* (Puechmaille et al, 2005; Puechmaille and Petit, 2007; Zarzoso-Lacoste et al., 2018). It should also be noted that out of

396 full genotypes obtained in this study, only one instance of potential misassignment was seen. Thus, this is a rare occurrence, probably due to the presence of very closely related individuals, and is unlikely to present a significant source of error in the identification of individuals or in the estimation of sex ratios.

The previous assumption used in estimates of Ireland's *R. hipposideros* population was that approximately 25% of adult bats counted in maternity roosts were males. The data from this study show that the sex ratios of *R. hipposideros* roosts in Ireland vary much more widely in reality, with the proportion of males ranging from as low as 14.2% to as high as 74.3%. Interestingly, in a study of *R. hipposideros* roosts in a small area of northern France, Zarzoso-Lacoste et al. (2018) also found a wide variation in the proportion of males present in maternity roosts, ranging from 0% to 50%, but the variation was not as great as seen in this study. Zarzoso-Lacoste et al. (2018) also found that the sex ratio of lesser horseshoe bats in the colonies they studied changed over the course of the summer, with a decrease in the proportion of males seen at a majority (52%) of colonies. It is possible that changes in the sex ratio of individuals also occur at Irish lesser horseshoe bat colonies during the summer period, and thus the results of this study only provide a snapshot of the sex ratio of each of the colonies studied. However, although further work is required to determine the extent to which lesser horseshoe bat sex ratios within colonies change over the course of the year, the data in this study still provide a far better basis for a national population estimate for this species than the previously used sex ratio estimates.

In order to obtain a more robust population estimate for Ireland's lesser horseshoe bat population, further work will be required to obtain more sex ratio data from a greater number of colonies. The work involved in non-invasive sampling and genetic analysis as used in this study requires a substantial investment, in terms of cost, time and skilled labour. However, the other alternative, of trapping all of the bats present in a summer colony to determine their sex, is not viable, for several reasons. Firstly, this method equally involves a large investment of time and labour, by people skilled in trapping and handling bats. Secondly, this method would involve serious disturbance to the bats present in any colony sampled thus, which would have implications for the animals' welfare. Lesser horseshoe bats are known to be highly sensitive to disturbance: in a pilot study to examine the possibility of using translocations of individuals to boost the populations of small colonies of both *R. ferrumequinum* and *R. hipposideros* in Switzerland, two out of seven (29%) translocated lesser horseshoe bats (which had been trapped within their roost) died within two days due to shock (Weinberger et al., 2009).

Although a study by Bontadina et al. (2002) was able to obtain sex typing data by trapping lesser horseshoe bats at a short distance (10 - 30 m) from their summer roost and reported no casualties in the process, only a minority (90 out of 300) of the colony's population was sampled in this way. Thus, directly trapping bats within their roost would be the only way of guaranteeing capture of the majority of a colony's population, but would carry with it the serious risk of causing a high number of fatalities due to shock, or the abandonment of the roost by the resident bats. As another obstacle, due to this risk of serious disturbance an NPWS licence to trap lesser horseshoe bats within their roost in Ireland would be highly unlikely to be granted, as the damage which would be caused could not be justified by the data gathered. Therefore, the non-invasive genetics approach outlined in this study, despite some drawbacks, represents the only realistic approach towards gathering the sex ratio data presented here.

The findings of this study will have implications for future population estimates for *R. hipposideros* in Ireland, as it is evident that the proportion of males present in this species' roosts here is substantially higher than previously assumed. The sex ratio data obtained in this study resulted in a significantly lower national population estimate for this species compared the most recent published estimate.

4.5 Conclusion

- This study successfully applied non-invasive DNA techniques for the species identification, sex typing and individual identification of *R. hipposideros* to assess the proportion of males present in maternity roosts of this species in Ireland.
- The results of this study show that the proportion of males in maternity roosts is substantially higher than previously assumed, and will have an impact on future population estimates for this species.
- The technique used in this study could be used more widely in Ireland in order to provide more data on this question, and could be expanded to other countries in Europe to provide data on *R. hipposideros* populations there also.

Chapter 5

Examining the population structure of the lesser horseshoe bat in Ireland

5.1 Introduction

The lesser horseshoe bat has the most restricted range of Ireland's resident bat species, present only in six counties on the west coast, and also has the smallest estimated population, currently thought to be approximately 14,000 individuals (Roche et al., 2014). The geographic location of the Irish lesser horseshoe bat population leaves it extremely isolated from its nearest neighbouring population in Wales, being separated by a distance of 250 kilometres and a significant barrier, the Irish Sea.

In addition to its isolation and small size, the lesser horseshoe bat populations of Ireland and Great Britain have been found to be the least genetically diverse in the entire range of this species in Europe and western Asia (Dool et al., 2013). Due to its small size, extreme isolation and lack of genetic diversity, the Irish lesser horseshoe bat population is at risk of developing inbreeding depression. In turn, this poses a risk of the local extinction of the lesser horseshoe bat in Ireland.

However, the Irish population does not inhabit a continuous range, which may pose a risk of inbreeding. Using analysis of microsatellite and mtDNA markers, Dool et al. (2016) found that the population appeared to be split into two isolated groups, a "south range" consisting of the colonies located in Cork and Kerry and a "north range" consisting of the colonies in Limerick, Clare, Galway and Mayo. These groups are not separated by a major physical barrier or apparent lack of suitable habitat, but a lack of suitable roosting sites may be implicated.

Within the "north range" group, further geographical subdivisions are apparent with the potential to present a barrier to interbreeding. The small subpopulation in Limerick, a crucial potential link between the larger subpopulations in Cork/Kerry and Clare, has been in decline for several decades and is now mainly limited to small cluster of colonies in the centre of the county (Roche, 2001; Roche et al., 2015). Although the River Shannon presents an obvious geographic barrier to the movement of bats between this group and the large and genetically diverse cluster of colonies in Clare, Dool et al. (2016) did not find any genetic indication of isolation in the Limerick subpopulation.

Dool et al. (2016) also suggested that there appeared to be some genetic structure which indicated that the northernmost lesser horseshoe bat colonies in north Galway (i.e. north of Galway City) and Mayo were differentiated from the colonies further south. For a distance of

approximately 22 kilometres to the south of Galway City there are no recorded lesser horseshoe bat maternity roosts, and this region separates the small subpopulation of north Galway and Mayo from the larger subpopulation present in south Galway and Clare. This apparently uninhabited part of County Galway could represent a barrier to movement and gene flow for lesser horseshoe bats, as this is a sedentary species which generally travels less than 20 kilometres from its roost of residence, although the longest recorded dispersal distance was 153 km (Dietz et al., 2009). Such a lack of gene flow could indicate a further subdivision of the already fragmented Irish population.

The aim of this chapter is to examine the population genetics of the lesser horseshoe bat in the northern part of its range in Ireland using non-invasive sampling methods, with a particular focus on the "Galway gap", and to determine whether the north Galway-Mayo colonies form a genetically isolated subpopulation.

5.2 Materials and Methods

5.2.1 Sample collection

For this study, bat droppings were collected from roosts from across the lesser horseshoe bat's range in Ireland. Fresh bat droppings samples which were collected as part of the sex typing study (Chapter 4) at the roosts in Derrycreha, Courtney's Cottage, Curragh Chase, Toonagh, Garryland and Ballykine were used in this study. In addition, bat dropping samples were collected from another 25 known lesser horseshoe bat roosts by the author, Vincent Wildlife Trust staff and National Parks and Wildlife Service rangers, bringing the total number of sampled roosts to 31 (Table 5.1). Samples from these 25 sites were collected from bat droppings accumulated on the floor of each roost, which were then stored in universal containers and subsequently frozen at -20°C.

5.2.2 DNA analysis

From each site, 10-25 individual bat droppings were selected for DNA extraction, which was carried out as described in Section 2.2.1.2. DNA extracts were identified to species as described in Section 2.2.1.4, using the RhipCytbF/R real-time PCR primer set. The real-time PCR Ct value obtained from the species identification assay was used as a means of assessing the quantity of DNA present in each sample, as only samples with a Ct value of < 25 were considered for microsatellite genotyping analysis. Microsatellite genotyping was carried out as per section 4.2.4, using the microsatellite panel of seven loci shown in Table 4.7.

Table 5.1: Table of known lesser horseshoe bat roosts sampled for this study.

Site location	County	Grid reference (Irish Grid)	Collection date	Collector
Derrycreha	Cork	V 954 549	24/06/2016	A Harrington
Drumbohilly Lower	Kerry	V 779 620	02/12/2015	A Harrington
Releagh Bridge	Kerry	V 925 627	02/12/2015	A Harrington
Askive Wood	Kerry	V 711 657	02/12/2015	A Harrington
Caher Bridge	Kerry	V 964 729	02/12/2015	A Harrington
William King House	Kerry	W 033 740	02/12/2015	A Harrington
Courtney's Cottage	Kerry	V 974 857	01/06/2016	A Harrington
Curragh Chase House	Limerick	R 410 490	14/07/2016	A Harrington
Ahaclare	Clare	R 528 723	June 2016	D Lyons
Cullaun House	Clare	R 474 747	June 2016	D Lyons
Corbally	Clare	R 421 783	June 2016	D Lyons
Knockaskibbole	Clare	R 366 775	June 2016	D Lyons
Knockanean	Clare	R 370 784	June 2016	D Lyons
Toonagh	Clare	R 308 822	14/06/2016	A Harrington
Rylane	Clare	R 433 831	July 2016	K McAney
Lisduff	Clare	R 325 863	July 2016	K McAney
Dromore	Clare	R 348 872	July 2016	K McAney
Knockreddan	Clare	R 427 865	June 2016	S Biggane
Ballyallaban Stables	Clare	M 228 045	June 2016	S Biggane
Fiddaun	Galway	R 399 953	July 2016	K McAney
Garryland Lodge	Galway	M 412 039	28/06/2016	A Harrington
Ballylee Castle	Galway	M 481 060	14/06/2016	A Harrington
Cloghballymore	Galway	M 397 140	28/06/2016	A Harrington
Menlo Castle	Galway	M 284 278	28/06/2016	A Harrington
Ross House	Galway	M 178 375	20/6/2016	R Teesdale
Ballykine	Mayo	M 107 563	28/06/2016	A Harrington
Lough Mask House	Mayo	M 144 604	July 2016	J Higgins
Inismaine	Mayo	M 135 615	July 2016	J Higgins
Scalpnagot Cave	Mayo	M 161 624	July 2016	J Higgins
Bunnadober Mill	Mayo	M 162 627	July 2016	J Higgins
Towerhill Cottage	Mayo	M 202 754	28/05/2014	K McAney

5.2.3 Microsatellite data analysis

GenAlEx version 6.503 (Peakall and Smouse, 2006) was used to identify unique genotypes. In order to assess the level of genotyping errors, the data obtained was first examined using Micro-Checker version 2.2.3 (van Oosterhout et al. 2004), using default settings. This software was used to check the data for possible genotyping errors, including null alleles, stutter peaks, allelic dropout and false alleles, which could potentially affect subsequent analysis.

5.2.3.1 Genetic structure

The presence of population substructure within the set of *R. hipposideros* individuals was tested using the programme STRUCTURE 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). STRUCTURE uses Markov Chain Monte Carlo (MCMC) analysis, a form of clustering algorithm, to assign individuals to one of a specified number of populations based on allele frequency patterns, and assumes that the data are in Hardy-Weinberg and linkage equilibrium. Through numerous iterations (i.e. repeated calculations), the programme probabilistically assigns each individual within the dataset to a particular population cluster (Hubisz et al., 2009).

Prior to running the programme, a set of parameters is defined within the bounds of which it will assign individuals to populations. A range of numbers of potential populations within the data must be assigned (K), and the number of replicates to be carried out for each value of K. The number of iterations to be carried out during each replicate is also defined, as well as a "burn-in", discarding an initial set of iterations. An admixture model also allows individuals with mixed ancestry to be correctly assigned. The programme has a "LOCPRIOR" option, allowing prior sampling location information to assist the clustering process. For this study, the parameters used were K = 1-6 with five replicates for each value of K, a burn-in of 250,000 iterations with 750,000 subsequent iterations, and the admixture model and LOCPRIOR were applied. The run was also repeated without the LOCPRIOR option.

In order to select the most likely value of K, the output data was assessed using both the Ln Pr (X|K) method, and the Delta K method (designed by Evanno et al. (2005)), as recommended by Janes et al. (2017). These methods were carried out using Structure Harvester (Earl and van Holdt, 2012). Both methods are ad-hoc measures, based on examining the posterior probabilities generated by STRUCTURE. STRUCTURE plots were visualised using CLUMPAK (Kopelman et al., 2015).

As recommended by Janes et al. (2017), regional substructure was also examined within each of the main clusters identified in the large-scale population assessment. The parameters used were the same as those for the assessment of the entire population, with the exception that K values from 1-5 were used.

5.2.3.2 Descriptive statistics

Descriptive statistics were calculated for each geographically defined subpopulation, as indicated by the most likely value of K identified by STRUCTURE analysis. To examine finer scale variability, descriptive statistics were also calculated for each colony. For this analysis, sites from which fewer than 11 individuals had been sampled were merged with their nearest neighbour.

GenAlEx version 6.503 (Peakall and Smouse, 2012) was used to calculate the probability of identity (PI), the probability of identity of siblings (PI_{SIB}), observed (H_O) and expected (H_E) heterozygosity, and to identify deviations from Hardy-Weinberg equilibrium (HWE). The number of alleles (N_A) and number of effective alleles (N_E) per locus were calculated for subpopulations only. Heterozygosity is the overall proportion of individuals in a population which are heterozygotes at a particular locus, and can be used as a measure of the level of genetic variability within the population (Rowe et al., 2017). Hardy-Weinberg equilibrium is a measure of how stable the frequencies of different alleles are for a particular locus. Deviation from Hardy-Weinberg equilibrium equates to an excess of either homozygotes or heterozygotes for a particular allele. This can indicate issues such as inbreeding, undetected population substructure, hybridisation between populations, or genotyping errors such as null alleles (Rowe et al., 2017).

Allelic richness (A_R) and the inbreeding coefficient (F_{IS}) were calculated for individual roosts using Fstat version 2.9.3.2 (Goudet 2001). Fstat was also used to test the data at colony level for genotypic linkage disequilibrium, where alleles at different loci are non-randomly associated.

5.2.3.3 Genetic differentiation

F_{ST} values provide a measure of the level of inbreeding in a subpopulation relative to the entire population, and can be used as an indicator of the level of subpopulation differentiation (Rowe

et al., 2017). Pairwise F_{ST} values were calculated for subpopulations identified by STRUCTURE analysis and for sampled colonies using Fstat, which uses Weir and Cockerham's (1984) method to remove bias with respect to sample size. The significance was tested using 1000 randomisations and applying a Bonferroni correction.

5.2.3.4 Principal co-ordinate analysis

As an alternative approach to examining potential population structure, a series of principal co-ordinate analyses (PCoA) were carried out using GenAlEx. This analysis is based on genetic distance (Nei, 1978), and allows the plotting of major patterns within a multivariate dataset, e.g. multiple loci and samples. PCoA was performed for the entire dataset for both individuals and for colonies. Where STRUCTURE analysis identified distinct subpopulations, PCoA was also carried out separately for the colonies located within these subpopulations.

The genetic distance matrix created for PCoA was also analysed by Analysis of Molecular Variance (AMOVA), which made it possible to compare genetic variation between and within the populations to determine the level of population differentiation present in the data.

5.2.3.5 Isolation by distance

Isolation by distance (IBD) was a phrase used by Wright (1943) to describe a pattern of increasing genetic divergence in a species with geographic distance, which may be due to geographic restrictions or the limited dispersal ability of the species in question.

GenAlEx was used to carry out a Mantel test for isolation by distance of the dataset, with Nei's standard genetic distance (Nei 1972; Nei, 1978) and 999 permutations. Latitude and longitude data was provided for each colony sampled in order to calculate the geographic distance. Tests for isolation by distance were carried out for colonies within the entire population, and for colonies within the three main subpopulations identified by the STRUCTURE analysis.

5.2.3.6 Relatedness within populations

As analyses to assess the presence of population structure may be affected by the presence of related individuals within a dataset, the level of relatedness of *R. hipposideros* individuals within each colony was estimated using the pairwise relationship coefficient "r" from Lynch

and Ritland (1999) in GenAlEx. Expected values for r range from 0 for completely unrelated individuals to 0.5 for parent-offspring or full sibling pairs. The mean across all populations was randomised and bootstrapped (999 permutations) to iteratively calculate relatedness.

5.3 Results

5.3.1 Sampling success

The success of sampling and species typing of DNA samples for Derrycreha, Courtney's Cottage, Curragh Chase, Toonagh, Garryland and Ballykine has been discussed in detail in Chapter 4, as has the microsatellite genotyping of samples from Derrycreha, Toonagh and Ballykine. All of the unique genotypes identified in Chapter 4 were included in this analysis. In addition to these, 15-22 of the highest quality DNA samples from Courtney's Cottage, Curragh Chase and Garryland were subjected to microsatellite genotyping for this study, which yielded 15 unique genotypes from both Courtney's Cottage and Curragh Chase, and 21 from Garryland.

Of the samples collected from the 25 additional roosts, ten sites yielded bat droppings of poor quality. For some sites, the droppings that had been collected were visibly of poor quality (i.e. either damp and crumbly or turned to dust) and so DNA samples were not extracted. For DNA samples from other sites, testing with the real-time PCR species identification assay showed that the quantity of DNA present (as measured by Ct value) was not sufficient for microsatellite genotyping. From the remaining 15 roosts, an attempt was made to obtain genotypes from a minimum of ten DNA samples, but in some cases the number of bat droppings in each sample which were suitable for DNA extraction was less than ten. In total, 366 unique genotypes were obtained from 21 colonies across the species' range. A summary of the number of genotyped DNA samples and the number of identified individuals from each roost is shown in Table 5.2, and a map of the relevant roosts is shown in Fig. 5.1. The unique genotypes obtained are listed in Appendix 6.

Table 5.2: Summary of lesser horseshoe bat colonies from which genotyping data was successfully obtained, with the number of unique genotypes for each site. Colonies are numbered from south to north.

No.	Site	County	No. genotypes obtained	No. individuals
1	Derrycreha	Cork	155	64
2	Courtney's Cottage	Kerry	15	15
3	Curragh Chase	Limerick	16	15
4	Cullaun House	Clare	10	9
5	Corbally	Clare	10	9
6	Knockaskibbole	Clare	10	4
7	Knockanean	Clare	10	7
8	Rylane	Clare	20	20
9	Toonagh	Clare	127	53
10	Dromore	Clare	10	9
11	Ballyallaban Stables	Clare	10	8
12	Fiddaun	Galway	3	3
13	Garryland	Galway	22	21
14	Ballylee Castle	Galway	16	8
15	Cloghballymore	Galway	25	15
16	Menlo Castle	Galway	6	2
17	Ross House	Galway	25	15
18	Ballykine	Mayo	144	63
19	Lough Mask House	Mayo	6	2
20	Inishmaine	Mayo	8	6
21	Bunnadober Mill	Mayo	20	18
	Total			**366**

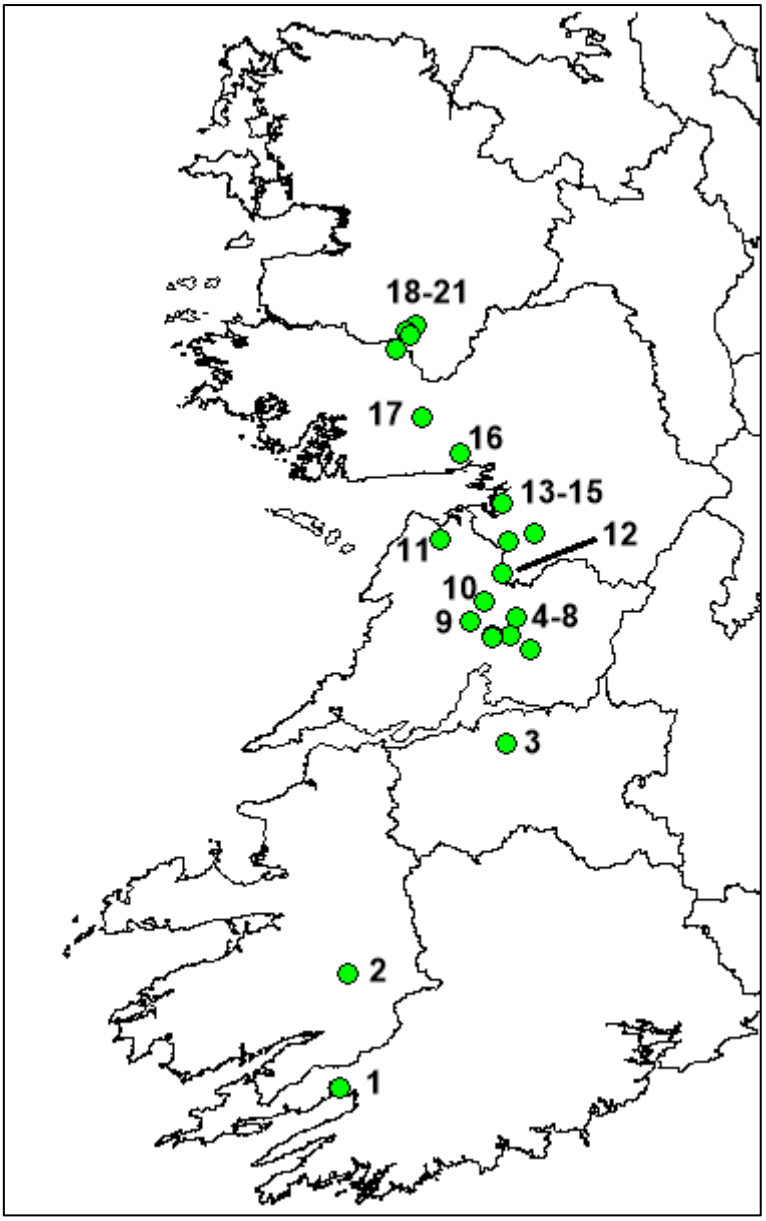

Figure 5.1: Map of sites from which microsatellite genotypes were obtained. Sites are numbered 1-21, as per Table 5.2.

5.3.2 Microsatellite data analysis

Analysis of unique genotypes using the programme Micro-Checker showed no evidence of stutter peaks, large allele dropout or false alleles. However, the programme did detect the potential presence of null alleles in individuals from two sites, where an excess of homozygous individuals at two different loci were found. At Rylane, an excess of homozygotes was detected for the RHD111 locus, while at Ballylee a similar excess was found for the RHD102 locus. No evidence of null alleles was found for any other locus in individuals from the other roosts.

All of the DNA samples genotyped from Rylane and Ballylee were of high quality (all of which had species assay Ct values < 20). Also, all of the replicated genotypes from these sites matched exactly, giving full consensus for the scored alleles. Therefore, it was concluded that the excess of homozygous individuals at the two loci at these roosts was more likely due to the effects of local inbreeding or isolation at these locations, and the presence of null alleles was discounted. All of the genotyped individuals were included in subsequent analysis.

5.3.2.1 Genetic structure

At a population-wide level, the STRUCTURE results appeared to show that K = 3 was the most likely number of genetic clusters present, but the number of geographically distinct clusters varied between simulations which ran LOCPRIOR and those which did not (Fig.s 5.2 and 5.3).

When LOCPRIOR was not used, the Delta K values showed that K = 3 was the most likely value, with other values of K showing much lower Delta K values (Fig. 5.4). In addition, the L(K) values showed a plateau beginning at K = 3, indicating that this represented the most likely value of K (Fig. 5.4). When LOCPRIOR was applied, the Delta K values showed that K = 2 was the most likely, while higher values of K were much less likely (Fig. 5.5). However, the L(K) graph again showed a plateau beginning at K = 3, indicating that this represented the true value of K (Fig. 5.5). Taken together, the majority of measures supported K = 3 being the most likely value of K.

The STRUCTURE plots showed the same patterns for runs carried out both with and without LOCPRIOR, for values up to and including K = 3. At K = 2, the individuals from Cork-Kerry, Limerick, Clare and South Galway formed a distinct cluster, with North Galway-Mayo a separate cluster. At K = 3, distinct clusters appeared in Cork-Kerry, Limerick-Clare-South Galway, and North Galway-Mayo. However, at higher levels of K (4-6), the simulations diverged. When the LOCPRIOR function was not used, the individuals in Cork-Kerry and

North Galway-Mayo remained as distinct clusters, while the individuals from Limerick, Clare and South Galway were subdivided into an increasing number of genetic clusters but with no evident geographic pattern (Fig. 5.2). In contrast, when the LOCPRIOR function was used, further geographically distinct clusters appeared: at K = 4, the individuals from the single colony in Limerick formed a highly distinct cluster; at K = 5, the individuals from one of the North Galway colonies (Ross House) also formed a distinct cluster; and at K = 6, the individuals from colonies in South Galway became partially differentiated from those in Clare (Fi.g 5.3). Despite their geographical distinctness, a certain level of admixture across all genetic clusters was observed for all values of K for both simulations (Fig.s 5.2 and 5.3).

Therefore, taking the evidence from the values for Delta K, L(K) and the geographic patterns produced for different values of K, it was concluded that K = 3 was the most likely scenario, splitting the Irish lesser horseshoe bat population into three geographically distinct subpopulations. These subpopulations consist of the Cork and Kerry group of colonies (colonies 1 and 2 in table 5.2), the colonies in Limerick, Clare and South Galway (colonies 3 to 15 in table 5.2), and the colonies in North Galway and Mayo (colonies 16 to 21 in table 5.2). These will be referred to as the southern, central and northern subpopulations, respectively.

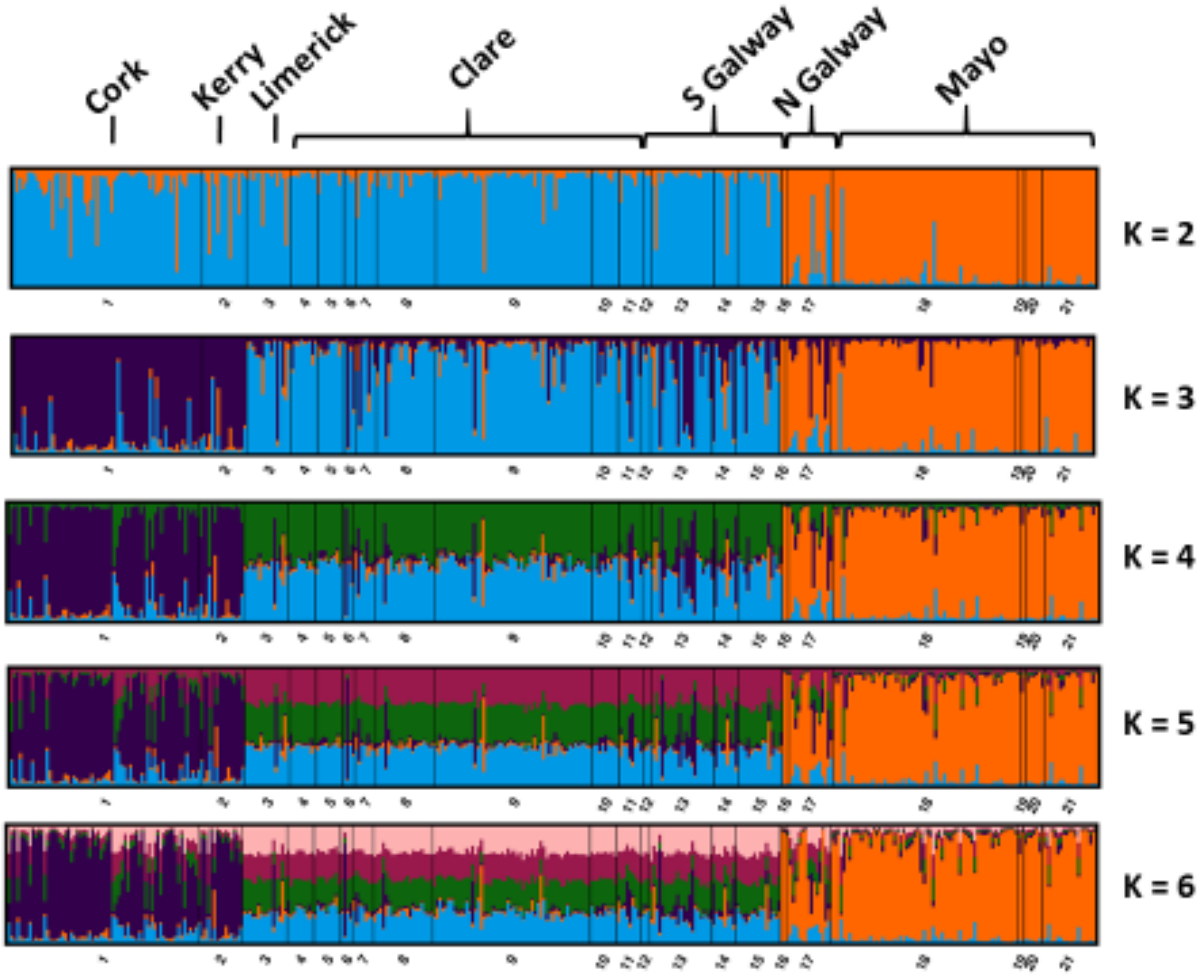

Figure 5.2: STRUCTURE plots for K = 2, K= 3, K =4, K = 5 and K = 6 for the entire set of individual genotypes from across the entire range of *R. hipposideros* in Ireland, where LOCPRIOR was not used.

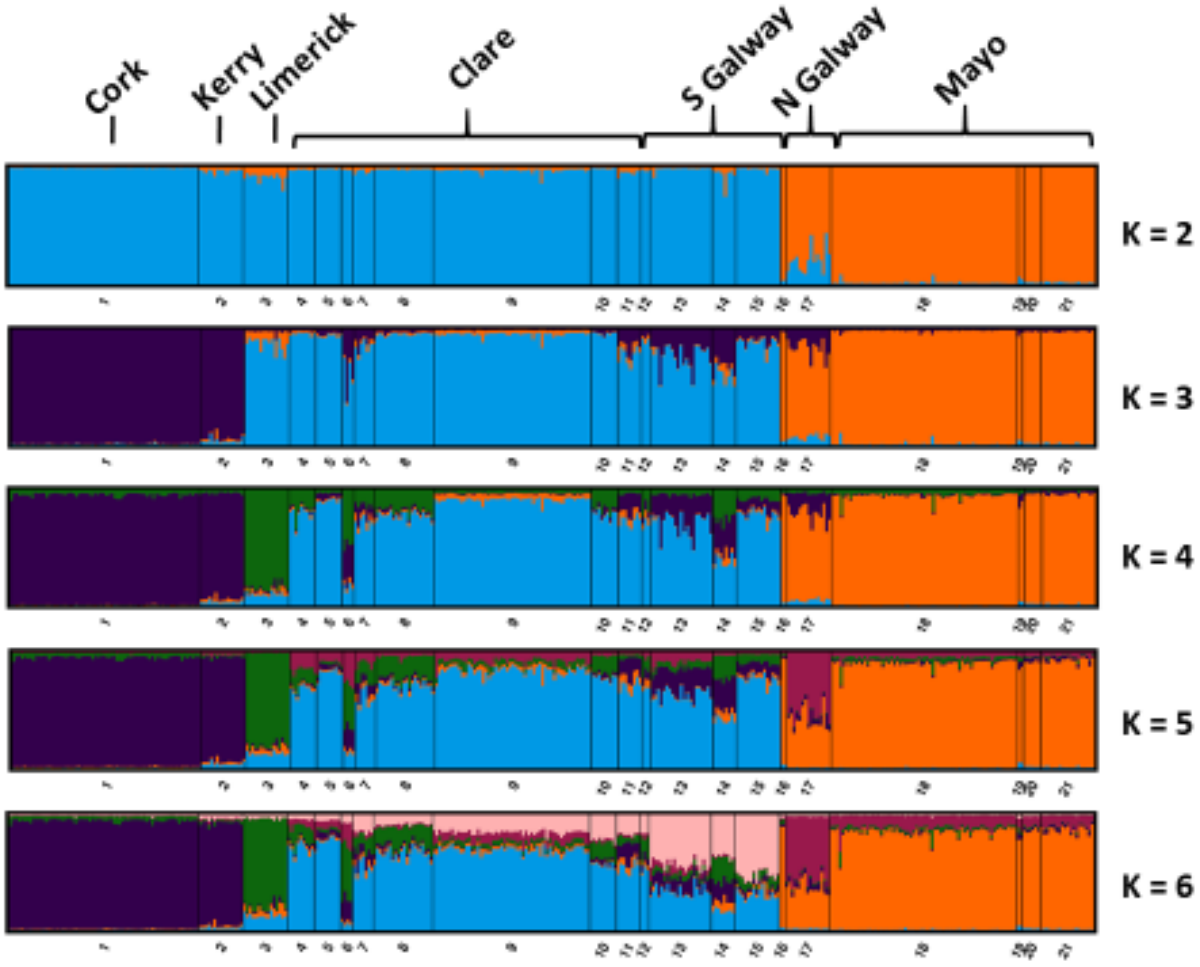

Figure 5.3: STRUCTURE plots for K = 2, K= 3, K =4, K = 5 and K = 6 for the entire set of individual genotypes from across the entire range of *R. hipposideros* in Ireland, where LOCPRIOR was used.

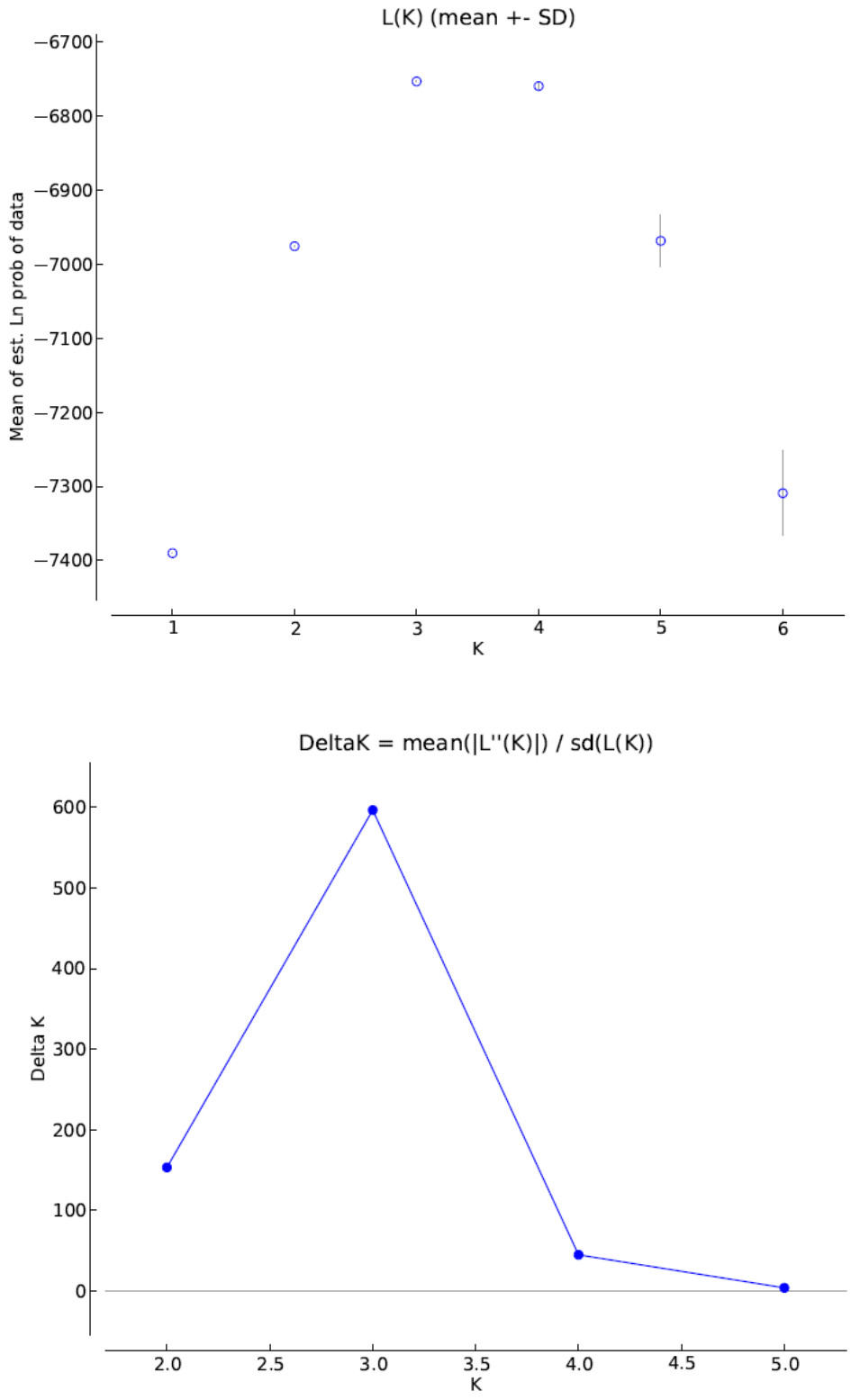

Figure 5.4: (above) Description of L(K) (±SD) across the five replicates and (below) Delta K values computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-6 and where LOCPRIOR was not used, for the entire set of individual genotypes from across the species' range in Ireland.

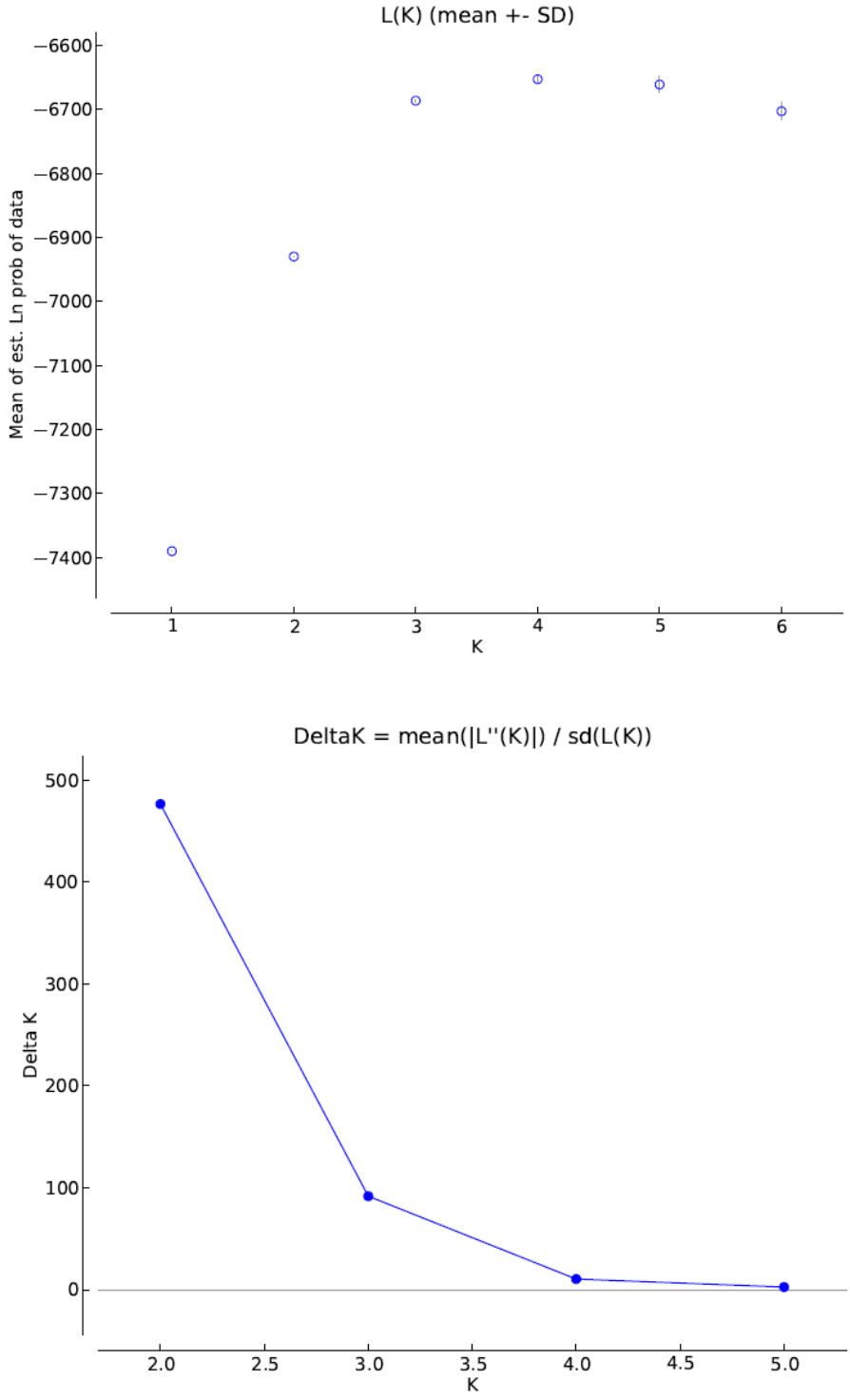

Figure 5.5: (above) Description of L(K) (±SD) across the five replicates and (below) Delta K values computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-6 and where LOCPRIOR was used, for the entire set of individual genotypes from across the species' range in Ireland.

Genetic structure was also investigated on a regional level, by separately analysing the individuals from each of the three geographical subpopulations identified above based on the clusters identified using STRUCTURE, where K = 3. In the southern subpopulation, no evidence of genetic structure could be found, both when LOCPRIOR was and was not used (Fig. 5.6). The L(K) graphs showed the highest value for K = 1 both when LOCPRIOR was and was not used, indicating that only a single genetic cluster was present (Fig. 5.7). In contrast, the Delta K graphs showed different peak values, with a peak at K = 2 when LOCPRIOR was used and a peak at K = 4 when LOCPRIOR was not used; however, it should be noted that Delta K values cannot account for the possibility that the most likely value of K is 1 (Fig. 5.8). The STRUCTURE plots showed that all individuals from both colonies displayed equal proportions of membership to each genetic cluster for all values of K, showing no geographic pattern. This implied that no true genetic structure was present and that the most likely value of K was 1 (Fig. 5.6).

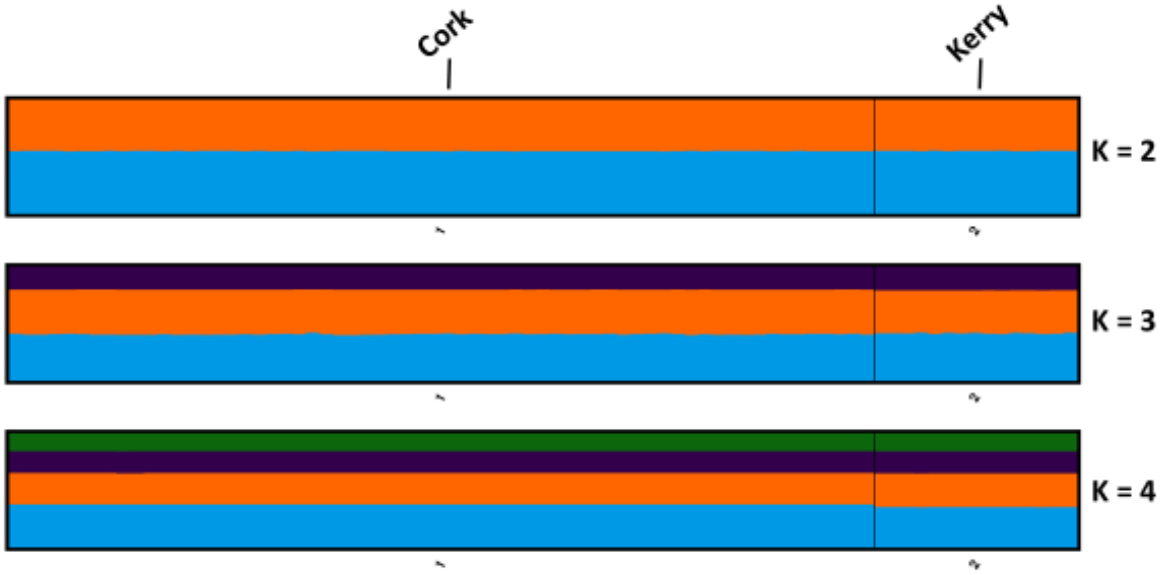

Figure 5.6: STRUCTURE plots for K = 2, K= 3 and K = 4 for the set of individual genotypes from the Southern subpopulation, where locprior was not used. An identical pattern was seen when LOCPRIOR was used.

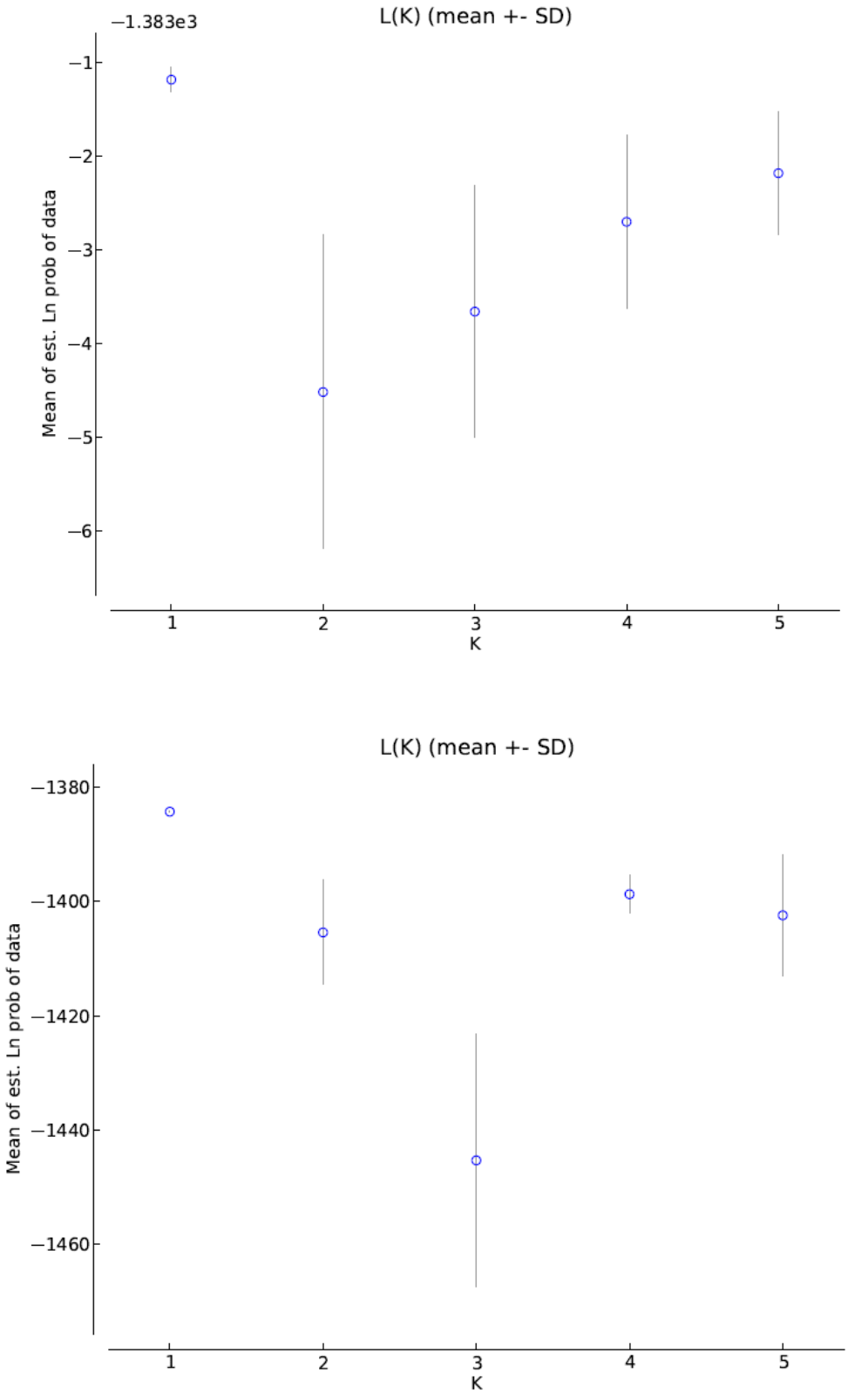

Figure 5.7: Description of L(K) (±SD) across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the southern subpopulation of *R. hipposideros*.

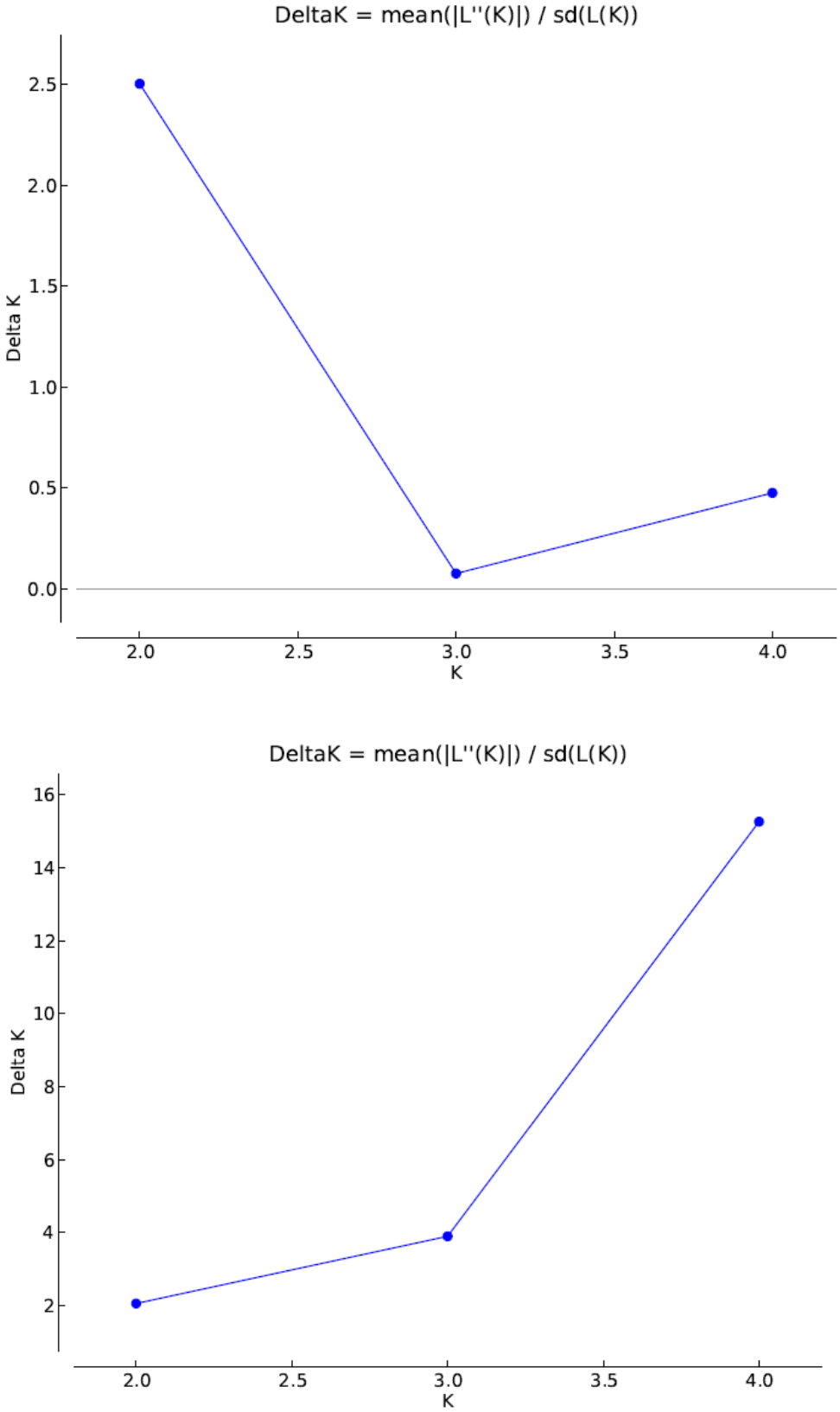

Figure 5.8: Description of Delta K values across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the southern subpopulation of *R. hipposideros*.

In the central subpopulation, evidence of potential further genetic structure was found, but only when the LOCPRIOR function was applied (Fig. 5.9). The L(K) graphs showed the highest value for K = 2 when LOCPRIOR was used, whereas when LOCPRIOR was not used the highest value obtained was for K = 1 (Fig. 5.10). The Delta K graph when LOCPRIOR was used again showed a weakly supported peak in values at K = 2, while the graph when LOCPRIOR was not used showed the highest value at K = 4 (Fig. 5.11).

The STRUCTURE plots showed different patterns of genetic structure depending on the use of LOCPRIOR. Where LOCPRIOR was not used, the STRUCTURE plots appeared very similar to those seen for the southern subpopulation, with equal subdivision of each individual among each putative genetic cluster and no discernible geographical pattern. However, when LOCPRIOR was used, a distinct geographical pattern was seen in the genetic structure (Fig. 5.9).

The difference seen between the two STRUCTURE runs indicates that weak genetic structure may be present, as LOCPRIOR is intended to assist STRUCTURE is assigning individuals to genetic clusters in such cases. When LOCPRIOR was used the most likely value of K was 2, showing that two geographically distinct genetic clusters were present. One cluster consisted of the Limerick colony and the second contained the other colonies in Clare and south Galway, with some admixture evident between the two clusters.

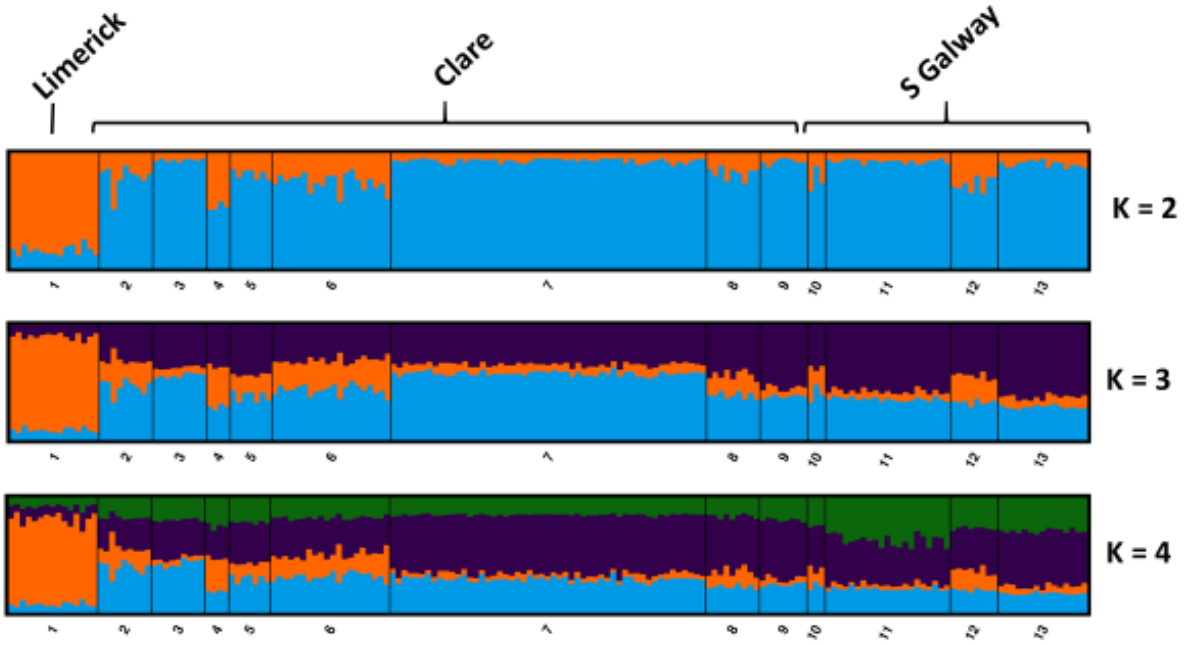

Figure 5.9: STRUCTURE plots for K = 2, K= 3 and K = 4 for the set of individual genotypes from the Central subpopulation, where LOCPRIOR was used.

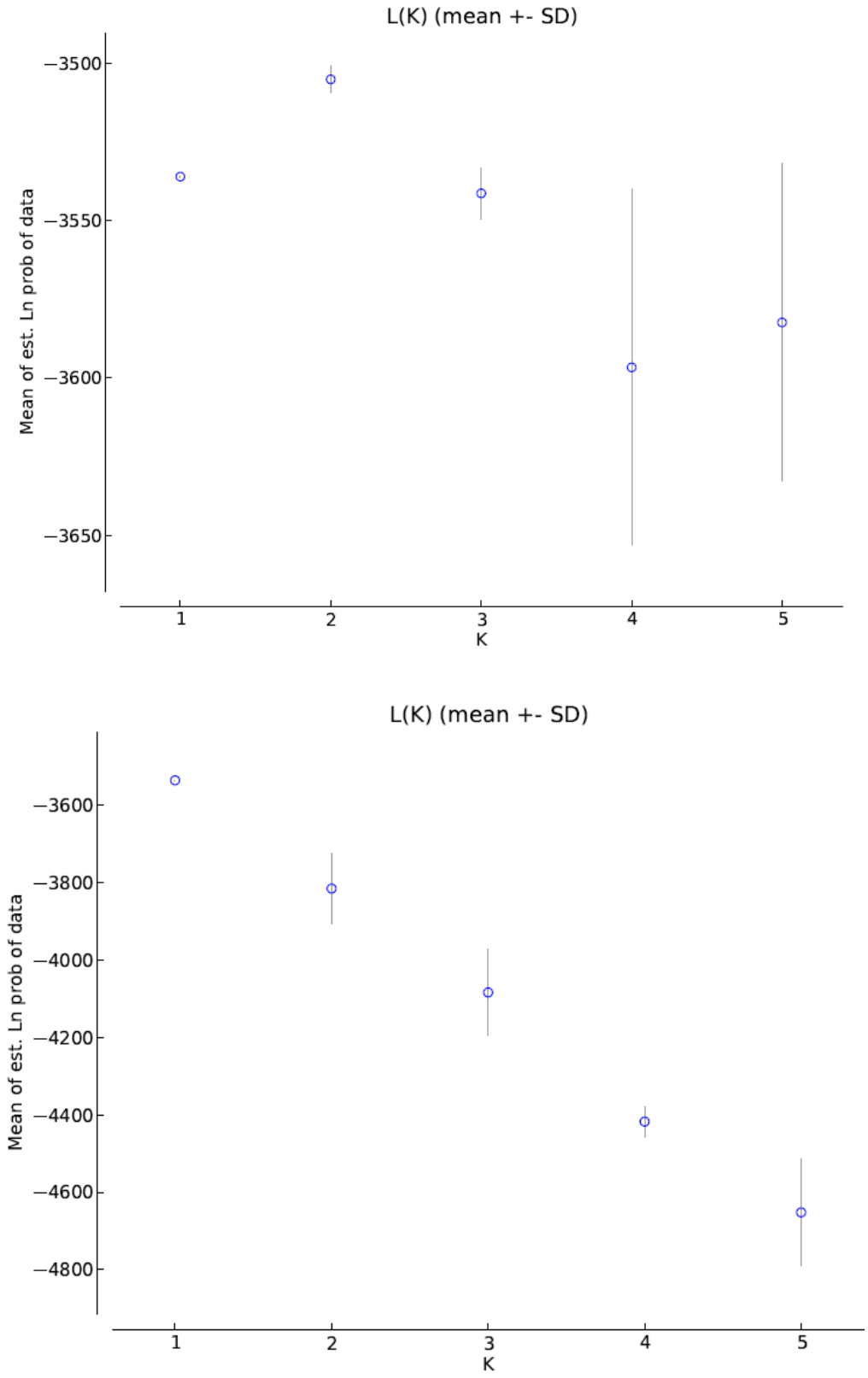

Figure 5.10: Description of L(K) (±SD) across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the central subpopulation of *R. hipposideros*.

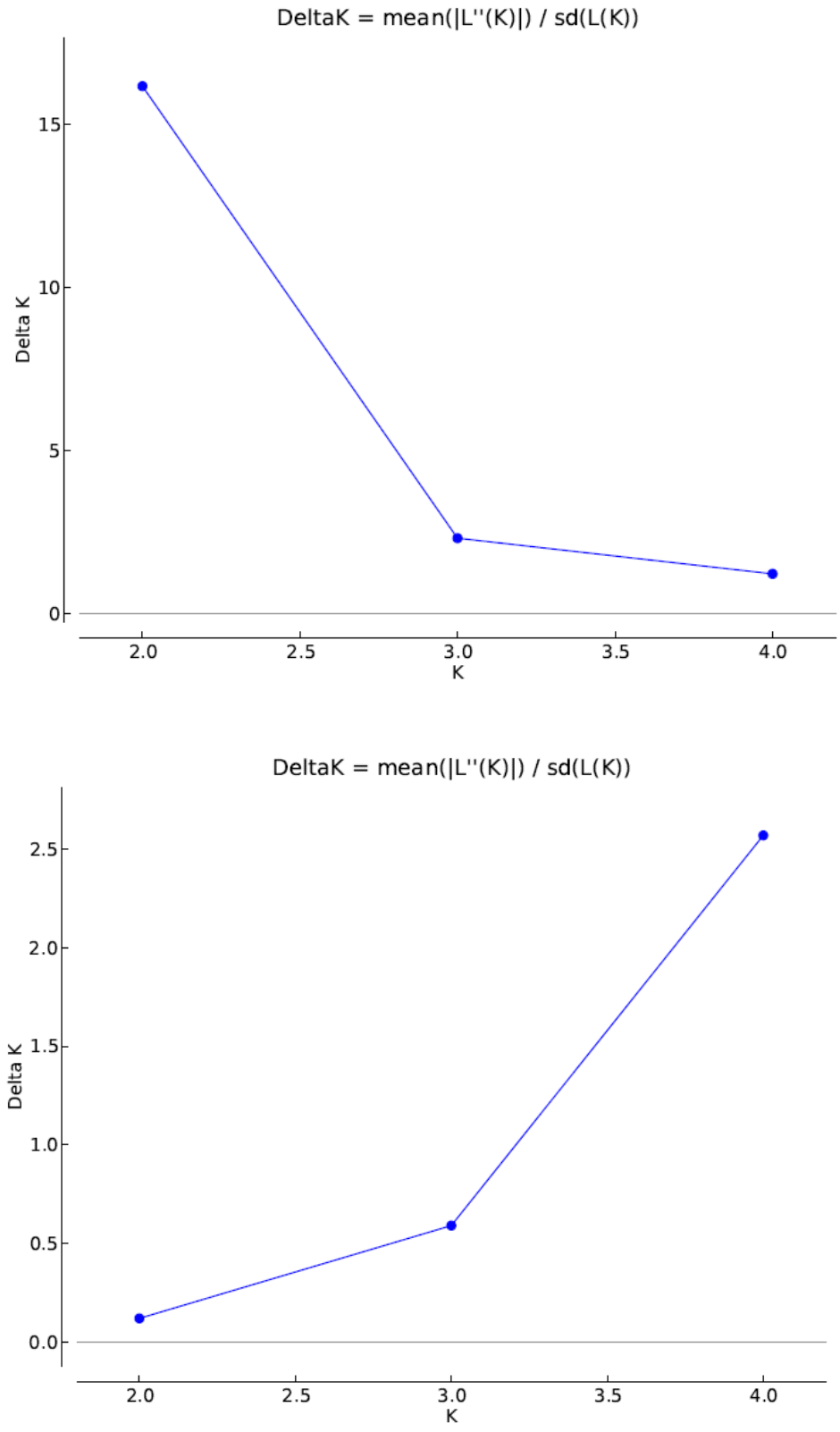

Figure 5.11: Description of Delta K values across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the central subpopulation of *R. hipposideros*.

Further potential genetic structure was also found in the northern subpopulation, but only when the LOCPRIOR function was applied (Fig. 5.12). The L(K) graphs showed the highest value for K = 2 when LOCPRIOR was used (but with a large error), whereas when LOCPRIOR was not used the highest value obtained was for K = 1 (Fig. 5.13). However, in this case both Delta K graphs showed a peak in values at K = 2 (Fig. 5.14).

The STRUCTURE plots showed different patterns of genetic structure depending on the use of LOCPRIOR. Where LOCPRIOR was not used, the STRUCTURE plots appeared very similar to those seen for the southern subpopulation, with equal subdivision of each individual among each putative genetic cluster and no geographical pattern. However, when LOCPRIOR was used, a distinct geographical pattern was seen in the genetic structure (Fig. 5.12).

The difference seen between the two STRUCTURE runs indicates that weak genetic structure may be present within this subpopulation. When LOCPRIOR was used the most likely value of K was 2, showing that two geographically distinct genetic clusters were present. One cluster consisted of the colony at Ross House in north Galway and the other contained all of the other colonies in the subpopulation, with little admixture evident. Unexpectedly, the colony at Menlo Castle appeared more similar to the Mayo colonies rather than Ross House. This may simply be due to the small number of individuals sampled from this site not allowing the true pattern of genetic structure to be discerned. Alternatively, it may imply that the Menlo Castle colony could be connected to the Mayo colonies via other currently unknown colonies.

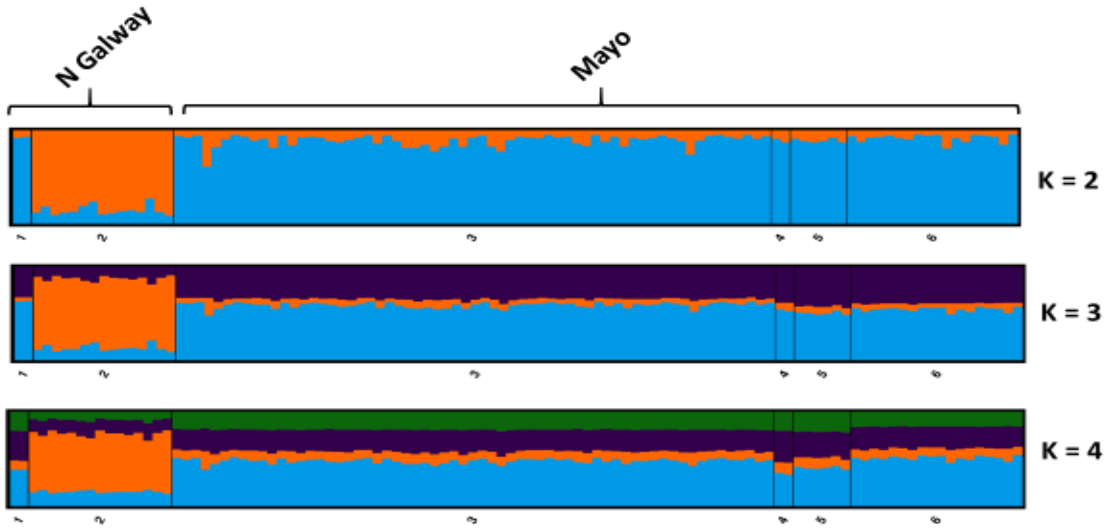

Figure 5.12: STRUCTURE plots for K = 2, K= 3 and K = 4 for the set of individual genotypes from the northern subpopulation, when LOCPRIOR was used.

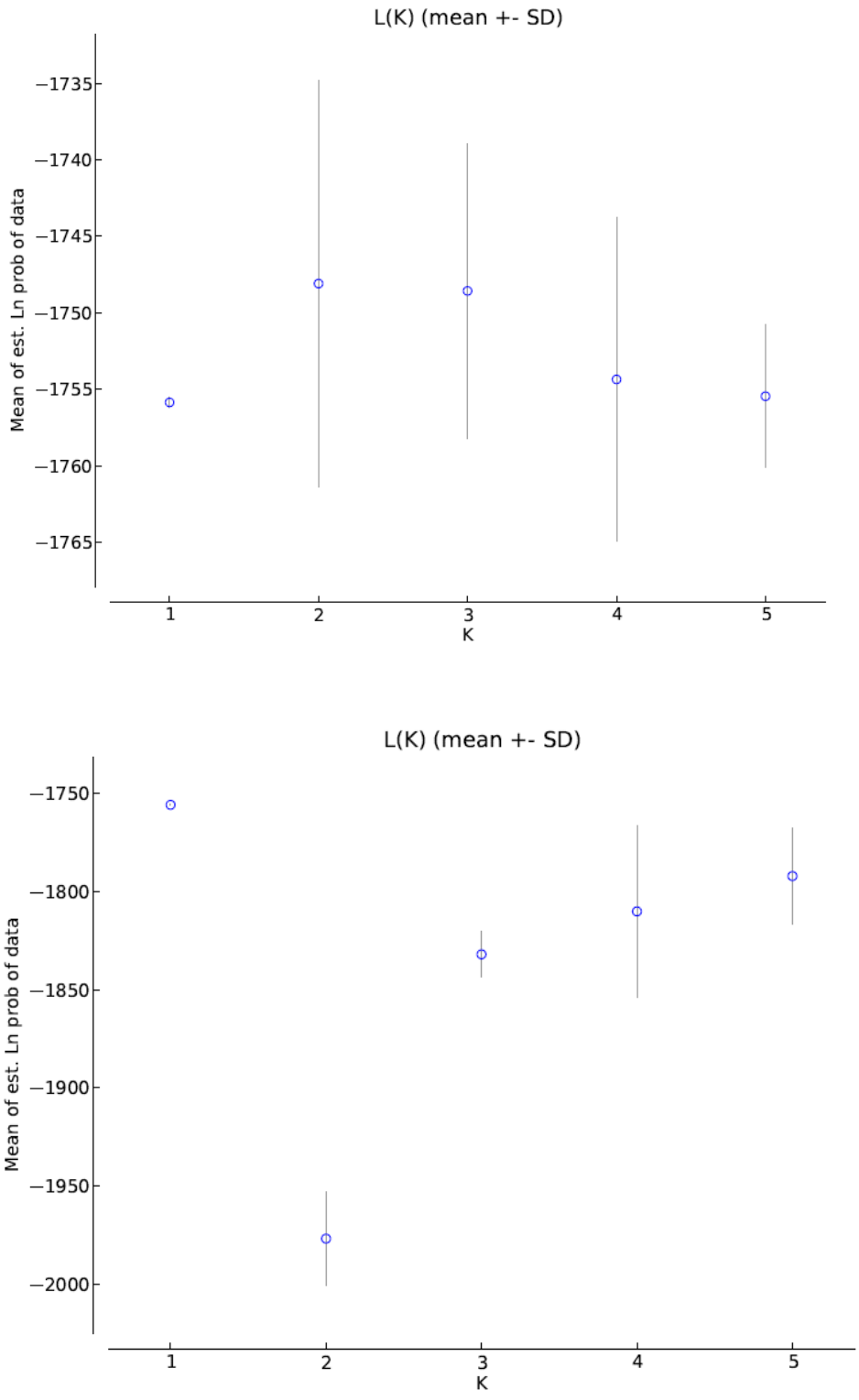

Figure 5.13: Description of L(K) (±SD) across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the northern subpopulation of *R. hipposideros*.

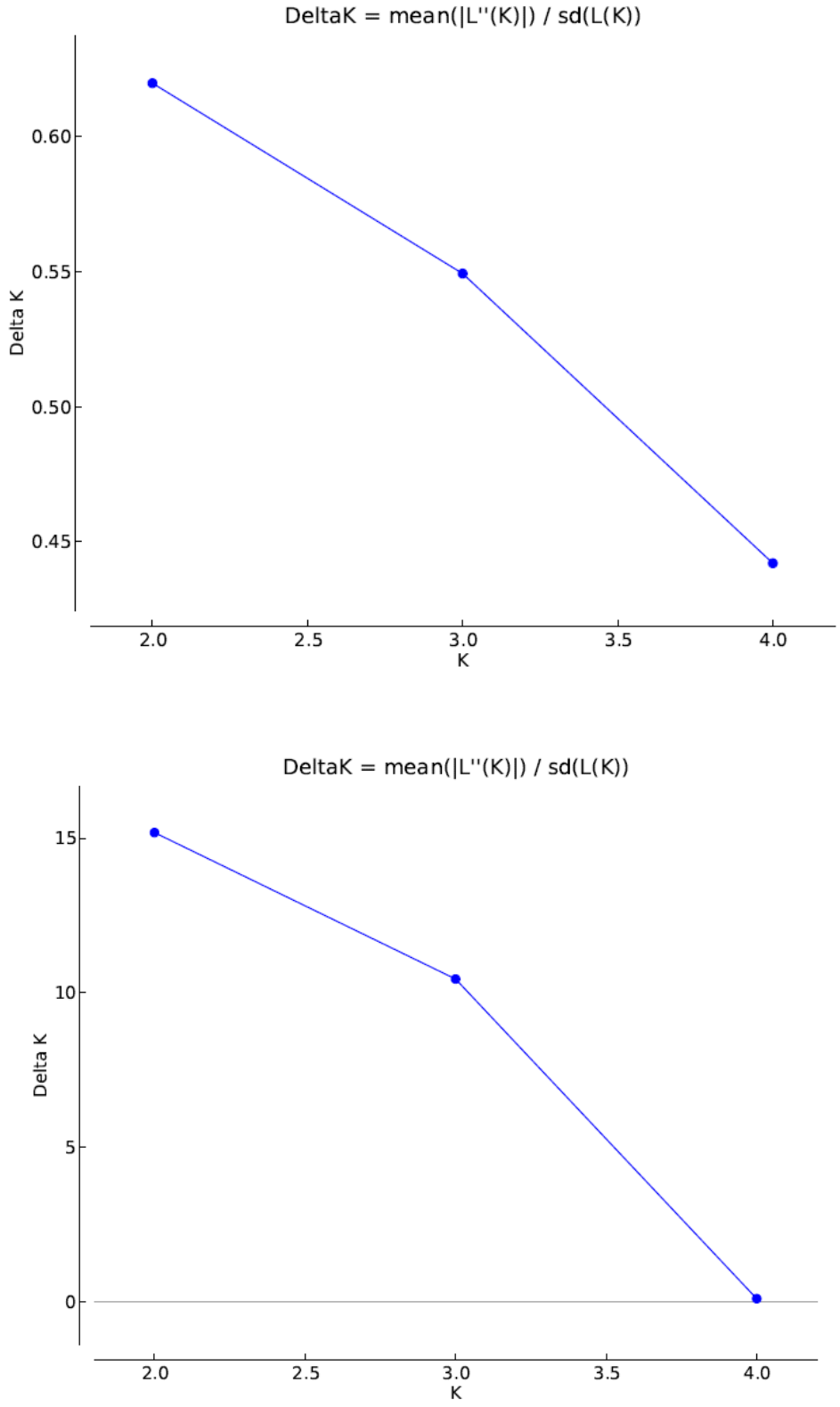

Figure 5.14: Description of Delta K values across the five replicates (above) where LOCPRIOR was used and (below) where LOCPRIOR was not used, computed by the software STRUCTURE (Pritchard et al., 2000) following Evanno et al. (2005), for K = 1-5, for individual genotypes from the northern subpopulation of *R. hipposideros*.

Overall, a substantial level of genetic structure was identified within the Irish lesser horseshoe bat population: three main subpopulations exist, two of which may in turn be subdivided by weak genetic structure. The geographical breakdown of these subpopulations and their respective subdivisions are shown in Fig. 5.15. The assignment of each individual colony to the three main subpopulations is summarized in Table 5.3.

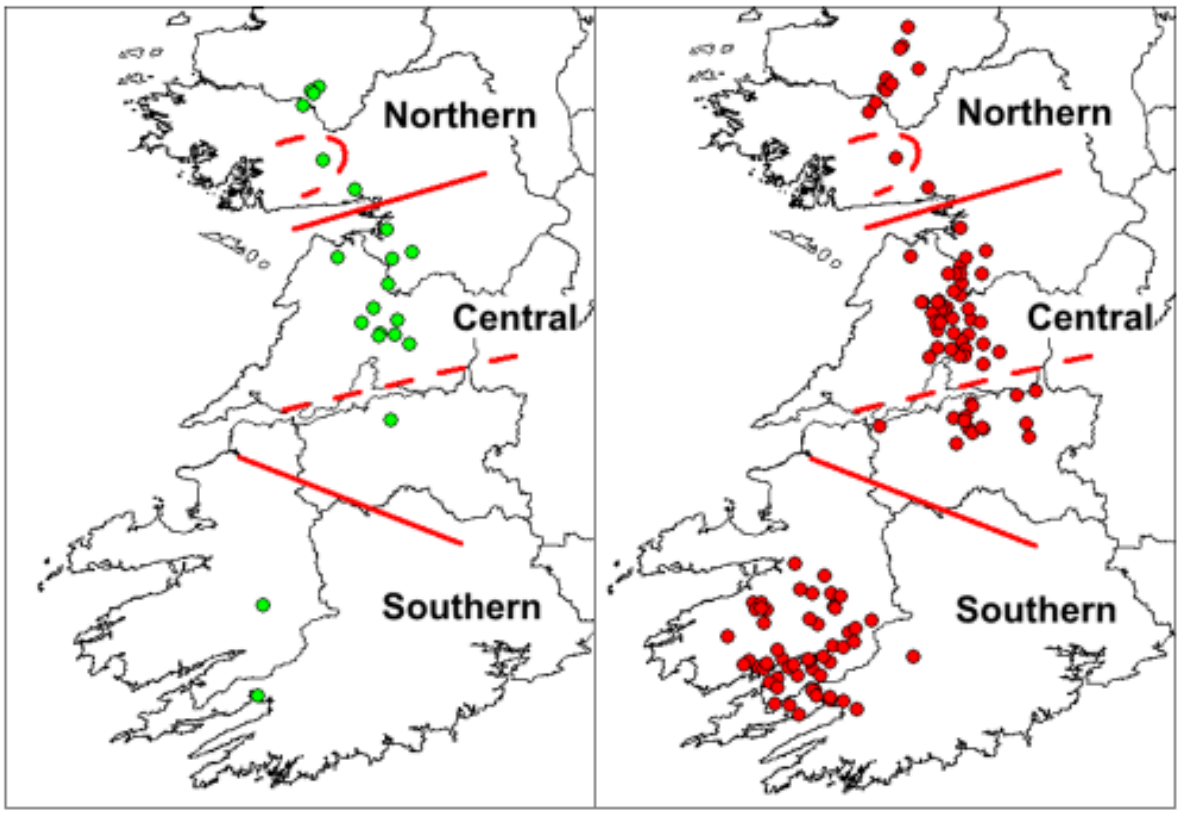

Figure 5.15: Map showing the genetic substructure found in the Irish lesser horseshoe bat population, showing (left) the sampled colonies and (right) all known *R. hipposideros* summer roosts. The three main subpopulations identified are labelled, with the geographic divisions between them indicated by solid red lines. The possible weaker genetic structure discovered within the northern and central subpopulations is indicated by dashed red lines.

Table 5.3: Assignment of individual colonies to subpopulations, based on the most likely scenario of K = 3.

No.	Site	County	Subpopulation
1	Derrycreha	Cork	Southern
2	Courtney's Cottage	Kerry	Southern
3	Curragh Chase	Limerick	Central
4	Cullaun House	Clare	Central
5	Corbally	Clare	Central
6	Knockaskibbole	Clare	Central
7	Knockanean	Clare	Central
8	Rylane	Clare	Central
9	Toonagh	Clare	Central
10	Dromore	Clare	Central
11	Ballyallaban Stables	Clare	Central
12	Fiddaun	Galway	Central
13	Garryland	Galway	Central
14	Ballylee Castle	Galway	Central
15	Cloghballymore	Galway	Central
16	Menlo Castle	Galway	Northern
17	Ross House	Galway	Northern
18	Ballykine	Mayo	Northern
19	Lough Mask House	Mayo	Northern
20	Inishmaine	Mayo	Northern
21	Bunnadober Mill	Mayo	Northern

5.3.2.2 Descriptive statistics

Following the STRUCTURE analysis in section 5.3.2.1, descriptive statistics were calculated for the three main populations identified, i.e. Southern (Cork and Kerry colonies), Central (Limerick, Clare and South Galway) and Northern (North Galway and Mayo) (Table 5.4).

Table 5.4: Descriptive statistics for the lesser horseshoe bats sampled, both for the entire sampled population, and according to each subpopulation identified by STRUCTURE analysis. Number of samples amplified per locus (N), number of alleles per locus (N_A), number of effective alleles per locus (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), Hardy-Weinberg expectations (HWE) and inbreeding coefficient (F_{IS}). * denotes deviation from Hardy-Weinberg equilibrium.

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2	Average
All sites								
N	366	366	366	366	366	366	366	366
N_A	2	6	7	9	7	8	12	7.3
N_E	1.3	2.0	2.9	5.0	4.3	4.8	8.8	4.2
H_O	0.216	0.459	0.637	0.784	0.732	0.719	0.754	0.614
H_E	0.218	0.499	0.660	0.798	0.768	0.792	0.886	0.660
HWE	0.869	0.000*	0.392	0.040*	0.016*	0.048*	0.000*	0.195
South								
N	79	79	79	79	79	79	79	79
N_A	2	4	6	7	7	6	7	5.6
N_E	1.2	1.5	3.7	4.4	4.9	4.3	2.6	3.2
H_O	0.203	0.304	0.759	0.772	0.835	0.759	0.633	0.609
H_E	0.182	0.320	0.728	0.778	0.797	0.769	0.613	0.598
HWE	0.317	0.001*	0.977	0.698	0.792	0.853	0.922	0.651
F_{IS}								-0.013
Central								
N	181	181	181	181	181	181	181	181
N_A	2	6	6	9	6	8	12	7.0
N_E	1.5	2.0	2.7	5.6	3.7	3.9	6.4	3.7
H_O	0.337	0.497	0.580	0.867	0.702	0.646	0.796	0.632
H_E	0.315	0.488	0.623	0.822	0.729	0.742	0.843	0.652
HWE	0.355	0.251	0.184	0.720	0.272	0.383	0.063	0.318
F_{IS}								0.033
North								
N	106	106	106	106	106	106	106	106
N_A	2	4	4	5	6	7	10	5.4
N_E	1.0	2.0	2.4	2.7	3.5	5.4	5.7	3.3
H_O	0.019	0.509	0.642	0.651	0.708	0.811	0.774	0.588
H_E	0.037	0.510	0.577	0.630	0.715	0.816	0.823	0.587
HWE	0.000*	0.858	0.574	0.879	0.067	0.398	0.000*	0.397
F_{IS}								0.004

The average number of alleles per locus ranged from 5.4 in the Northern population to 7.0 in the Central population, while the number of effective alleles (N_E) ranged from 3.2 in the Southern population to 3.7 in the Central population. Average expected heterozygosity ranged from 0.587 in the Northern population to 0.652 in the Central population, and observed heterozygosity ranged from 0.588 in the Northern population to 0.632 in the Central population. When the entire Irish population as a whole was examined, five of the alleles appeared to be out of Hardy-Weinberg equilibrium (HWE), but when each of the three subpopulations were assessed on their own only one allele was out of HWE in the Southern population, none in the Central population and two in the Northern population. This may represent an example of the Wahlund effect, where analysing two distinct populations with differing allele frequencies which may be in Hardy-Weinberg equilibrium can reduce the overall heterozygosity. F_{IS} values ranged from -0.013 in the southern population to 0.033 in the central population.

The probability of identity (PI) ranged from 6.4×10^{-6} for the Northern population to 8.7×10^{-7} for the Central population, and the probability of identity for siblings (PI_{SIB}) ranged from 7.6×10^{-3} for the Northern population to 4.1×10^{-3} for the Central population (Table 5.5). The PI_{SIB} values obtained for all three populations for the full microsatellite panel of seven loci was below the level of 0.05 recommended by Schwartz and Monfort (2008) for adequate discrimination of individuals.

Table 5.5: Table of probability of identity (PI) and probability of identity of siblings (PI_{SIB}) for the entire set of individual *R. hipposideros* analysed, as well as for each subpopulation.

	South	**Central**	**North**	**Overall**
PI	4.0×10^{-6}	8.7×10^{-7}	6.4×10^{-6}	3.4×10^{-7}
PI_{SIB}	6.6×10^{-3}	4.1×10^{-3}	7.6×10^{-3}	3.5×10^{-3}

Summary statistics were also calculated for each lesser horseshoe bat colony sampled (Table 5.6). However, four colonies were merged for this analysis with the next nearest colony, as some sites had fewer than the minimum 11 individuals required. The colonies merged were: Knockaskibbole and Knockanean in Co. Clare (0.8 km apart), Fiddaun and Garryland in Co. Galway (8.7 km apart), Menlo Castle and Ross House in Co. Galway (14.8 km apart), and Lough Mask House and Inishmaine (1.4 km apart).

Table 5.6: Summary statistics for the lesser horseshoe bats sampled, both for the entire sampled population, and according to each subpopulation identified by STRUCTURE analysis. Number of samples amplified per locus (N), mean observed heterozygosity (H_O), mean expected heterozygosity (H_E), mean Hardy-Weinberg expectations (HWE), allelic richness (A_R) and inbreeding coefficient (F_{IS}).

No.	Colony	N	H_O	H_E	HWE	A_R	F_{IS}
1	Derrycreha	64	0.596	0.588	0.766	4.074	-0.005
2	Courtney's Cottage	15	0.667	0.615	0.736	4.357	-0.050
3	Curragh Chase	15	0.533	0.533	0.493	3.550	0.034
4	Cullaun House	9	0.651	0.649	0.675	4.185	0.056
5	Corbally	9	0.683	0.632	0.453	4.184	-0.021
6	Knockaskibbole & Knockanean	11	0.597	0.581	0.786	4.567	0.020
7	Rylane	20	0.671	0.622	0.515	4.202	-0.053
8	Toonagh	53	0.685	0.647	0.605	4.496	-0.048
9	Dromore	9	0.556	0.608	0.550	4.439	0.144
10	Ballyallaban	8	0.661	0.627	0.522	5.429	0.013
11	Fiddaun & Garryland	24	0.596	0.588	0.472	4.760	0.067
12	Ballylee	8	0.482	0.565	0.549	4.143	0.211
13	Cloghballymore	15	0.610	0.611	0.477	4.830	0.037
14	Menlo Castle & Ross House	17	0.546	0.574	0.558	3.841	0.079
15	Ballykine	63	0.596	0.582	0.466	3.802	-0.017
16	Lough Mask House & Inishmaine	8	0.536	0.511	0.694	3.429	0.019
17	Bunnadober	18	0.619	0.559	0.496	3.642	-0.079

Average expected heterozygosity ranged from 0.533 at Curraghchase to 0.649 at Cullaun House, and observed heterozygosity ranged from 0.482 at Ballylee Castle to 0.685 at Toonagh. Mean Hardy-Weinberg probability ranged from 0.453 at Corbally to 0.786 at Knockaskibbole and Knockanean. Few instances of deviation from HWE at individual loci were found, with deviations found at RHD2 at Cullaun House, Dromore and Ballykine, at RHD102 at Ballyallaban, and at RHD111 at Cloghballymore. In addition, RHC108b was found to be monomorphic at the three most northerly colonies- Ballykine, Lough Mask House-Inishmaine and Bunnadober. Values of F_{IS} (the inbreeding coefficient) ranged from -0.005 at Derrycreha to 0.211 at Ballylee Castle. Values of A_R (allelic richness) varied from 3.429 at Lough Mask House-Inishmaine to 5.429 at Ballyallaban. No evidence of genotypic linkage disequilibrium at colony level was found.

5.3.2.3 Genetic differentiation

The pairwise F_{ST} values between subpopulations were relatively low, but all were found to be significant (P= 0.05) (Table 5.7), with the overall F_{ST} = 0.076 (99% CI 0.048- 0.114) found to be significantly different from zero.

Table 5.7: Pairwise F_{ST} values between *R. hipposideros* subpopulations. All values were found to be significant (P ≤ 0.05).

	Central	North
South	0.062	0.1125
Central		0.0932

Pairwise F_{ST} values between colonies were also relatively low (Table 5.8). All pairwise comparisons between colonies from the southern and northern subpopulations showed significant differences, with values ranging from 0.0979 to 0.1435. Pairwise comparisons between colonies from the southern and central subpopulations were generally significant, with some exceptions (3 out of 22 comparisons were not significant), with significant F_{ST} values ranging from 0.0323 to 0.1304. Pairwise comparisons between colonies from the central and northern subpopulations were also mostly significant with some exceptions (3 out of 44 comparisons were not significant), with significant F_{ST} values ranging from 0.075 to 0.2089.

Pairwise FST values between colonies from the same subpopulation generally were not significant, with the exception of two colonies. Curragh Chase (colony number 3) was significantly different from five of the ten other colonies in the central subpopulation, while Menlo Castle and Ross House (colony number 14) differed significantly from one of the three other colonies in the northern subpopulation.

Table 5.8: Pairwise F_{ST} values between *R. hipposideros* colonies. Values underlined, in bold are significant (P ≤ 0.05). Colonies are numbered as per Table 5.5.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0.0022	**0.1237**	**0.1022**	**0.0773**	**0.0475**	**0.0777**	**0.0776**	**0.0695**	**0.0323**	**0.0537**	**0.0406**	**0.0776**	**0.1185**	**0.1151**	**0.1435**	**0.1348**
2		**0.1304**	**0.084**	**0.0801**	0.0615	**0.0813**	**0.0794**	**0.0797**	0.0341	**0.0502**	0.0503	**0.0864**	**0.0979**	**0.1032**	**0.1159**	**0.1115**
3			0.0471	**0.097**	0.0379	**0.0543**	**0.0774**	0.0594	0.0738	**0.0592**	0.0557	**0.0778**	**0.1671**	**0.143**	**0.2089**	**0.1802**
4				-0.006	0.0207	-0.003	0.0206	0.0215	0.0155	0.0194	0.0311	0.0625	**0.0919**	**0.1001**	**0.1071**	**0.1107**
5					0.0105	0.0018	-0.0014	0.0093	-0.0142	0.0172	0.0188	0.0339	**0.0913**	**0.1098**	0.1269	**0.1227**
6						0.0108	0.0145	-0.0073	-0.0045	0.0033	0.0358	0.0104	**0.075**	**0.0836**	**0.1183**	**0.1136**
7							0.0056	0.0064	-0.0025	0.0191	0.0145	0.0311	**0.108**	**0.1009**	**0.1291**	**0.1239**
8								0.0068	-0.0173	0.0172	0.0239	0.0238	**0.0915**	**0.0938**	**0.116**	**0.1165**
9									-0.0188	0.014	0.0261	0.0062	**0.1128**	**0.1106**	0.1492	**0.1446**
10										-0.0089	-0.0056	-0.0029	**0.0829**	**0.0768**	**0.1058**	**0.1094**
11											0.0173	0.0088	**0.0986**	**0.1082**	**0.1413**	**0.1336**
12												0.053	**0.1247**	**0.1159**	0.151	**0.1428**
13													**0.1182**	**0.1058**	**0.1686**	**0.1371**
14														**0.0394**	0.0435	0.0415
15															0.0143	0.0001
16																-0.0019

5.3.2.4 Principal co-ordinate analysis

In the principal co-ordinate analysis (PCoA) of individuals for the entire Irish population, axis 1 differentiated individuals from the northern subpopulation in the right-hand quadrants. Axis 2 differentiated individuals from the southern and central subpopulations. However, there was significant admixture between all three groups. The first two axes of this analysis explained 20.25% of the genetic variation seen (Fig. 5.16).

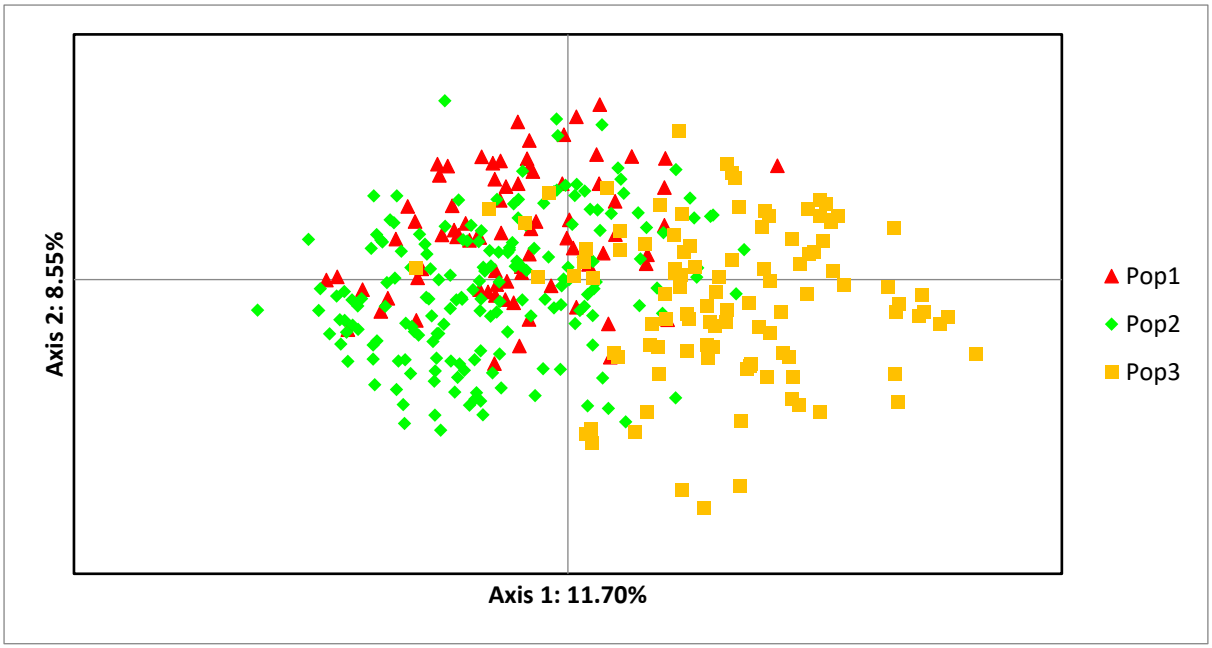

Figure 5.16: Principal co-ordinate analysis of *R. hipposideros* individuals from across Ireland, across seven loci and explaining 20.25% of genetic variation seen. Pop 1 = Southern subpopulation, Pop 2 = Central subpopulation, Pop 3 = Northern subpopulation.

A PCoA to visualise the clustering of colonies is shown in Figure 5.17, which explained 71.37% of the overall genetic variation. The colonies clearly grouped into three clusters representing the northern, central and southern subpopulations, matching the STRUCTURE output for K = 3. The AMOVA results showed that 86% of molecular variation occurred within populations and 14% occurred between populations.

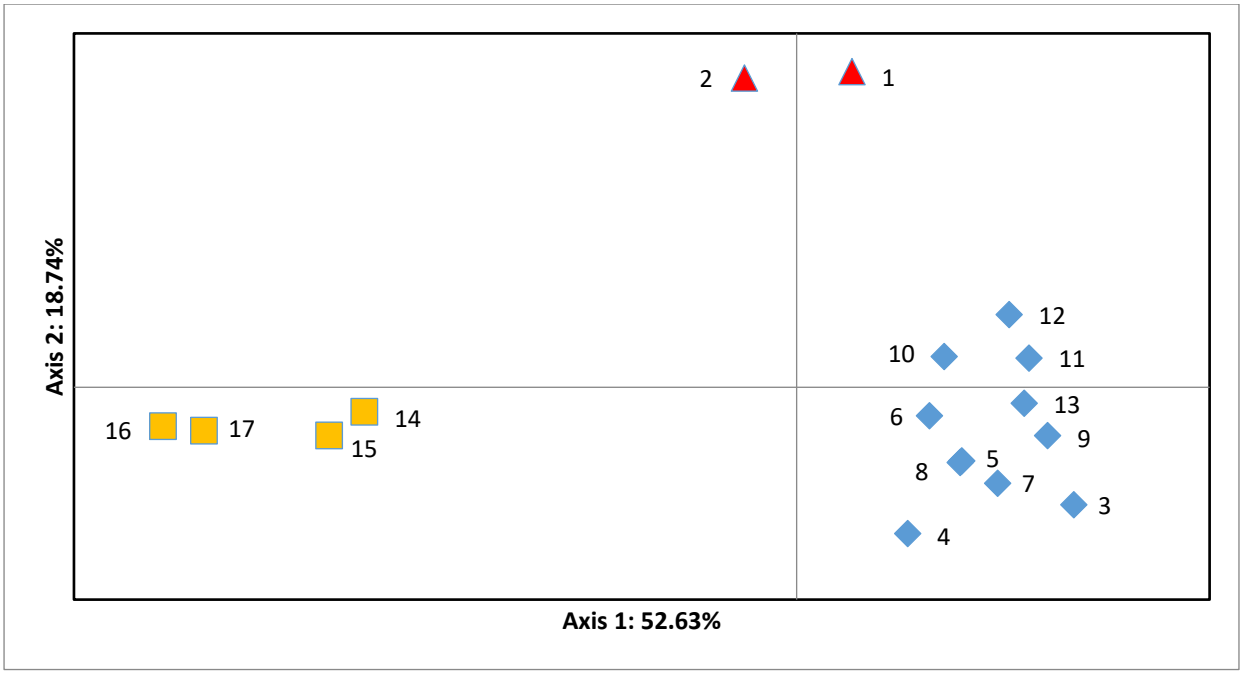

Figure 5.17: Principal co-ordinate analysis of *R. hipposideros* colonies across Ireland. This analysis explained 71.37% of the genetic variation observed. Colonies are numbered as per Table 5.5, with those from the southern subpopulation indicated by red triangles, central subpopulation by blue diamonds, and northern subpopulation by orange squares.

As only two colonies were sampled from the southern subpopulation, a more detailed PCoA by colonies of this subpopulation could not be carried out. However, PCoAs by colony of the other two subpopulations revealed further genetic differentiation, as was seen with the STRUCTURE analysis.

In the central subpopulation, the single colony at Limerick was highly differentiated from the other colonies in Clare and south Galway (Fig. 5.18). The most southerly colony in Clare (Cullaun House) and the most northerly colony in south Galway (Cloghballymore) were also somewhat differentiated. This analysis accounted for 77.54% of the genetic variation seen.

The PCoA for the northern subpopulation also showed significant structure, with the colonies in north Galway being highly differentiated from those in Mayo. There also appeared to be significant differentiation among the Mayo colonies (Fig. 5.19). This analysis accounted for 100% of the genetic variation seen.

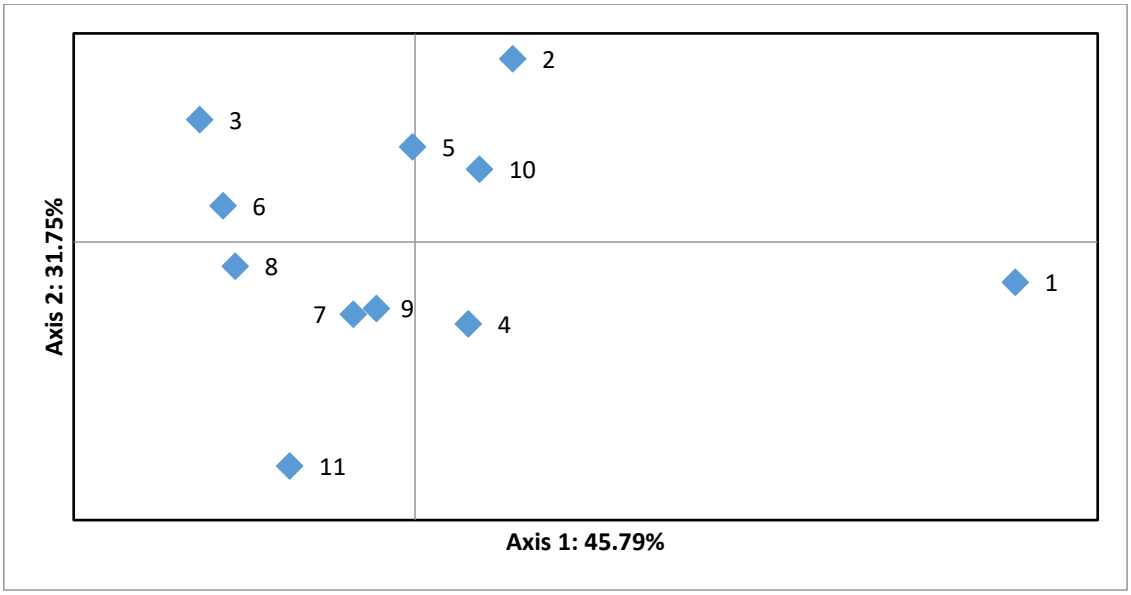

Figure 5.18: Principal co-ordinate analysis of *R. hipposideros* colonies in the central subpopulation. This analysis explained 77.54% of the genetic variation observed. Colonies are numbered as follows: 1. Curragh Chase, 2. Cullaun House, 3. Corbally, 4. Knockaskibbole and Knockanean, 5. Rylane, 6. Toonagh, 7. Dromore, 8. Ballyallaban, 9. Fiddaun and Garryland, 10. Ballylee, and 11. Cloghballymore.

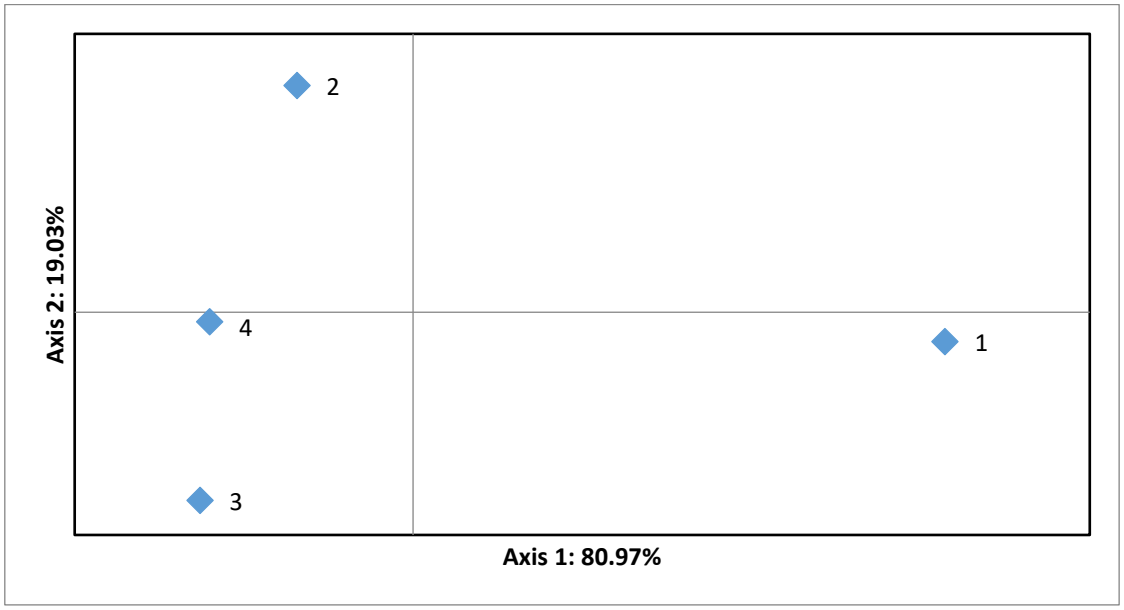

Figure 5.19: Principal co-ordinate analysis of *R. hipposideros* colonies in the northern subpopulation. This analysis explained 100% of the genetic variation observed. Colonies are numbered as follows: 1. Menlo Castle and Ross House, 2. Ballykine, 3. Lough Mask House and Inishmaine, and 4. Bunnadober.

5.3.2.5 Isolation by distance

The Mantel test carried out on the entire dataset showed a significant pattern of isolation by distance ($r = 0.165$, $P \leq 0.001$) (Fig. 5.20), which was expected due to the large distance between the most widely separated colonies in West Cork and Mayo (208 km). No pattern of isolation by distance was found in the southern subpopulation ($r = 0.022$, $P \leq 0.356$). However, significant isolation by distance was found within the central subpopulation ($r = 0.102$, $P \leq 0.001$) and the northern subpopulation ($r = 0.188$, $P \leq 0.001$).

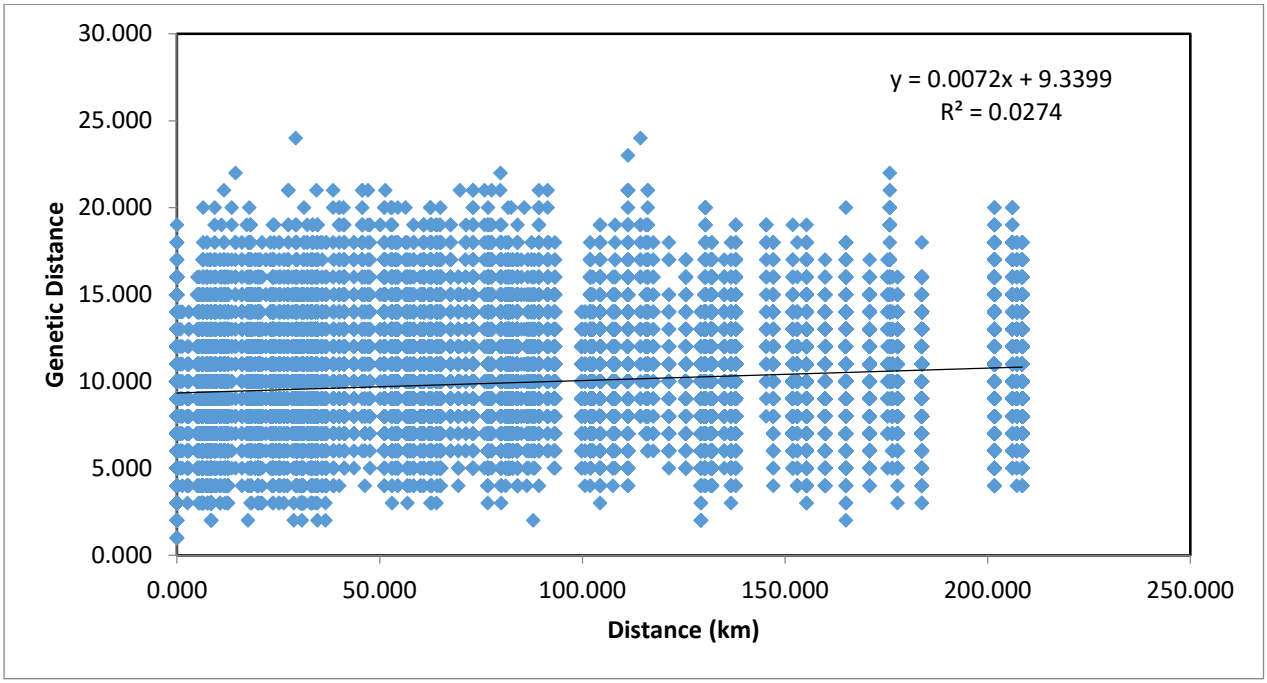

Figure 5.20: Mantel test showing observed pattern of isolation by distance (RXY = 0.166, P = 0.001). RXY indicates the correlation coefficient of the Mantel test, P(rxy-rand >= rxy-data) indicates the probability of positive autocorrelation.

5.3.2.6 Relatedness within populations

The pair-wise estimate of relatedness across the entire population showed that all of the colonies showed some evidence of related individuals, with the exception of one colony. Mean relatedness coefficient r values ranged from -0.009 in Ballyallaban (colony 10) to 0.107 in Bunnadober (colony 17) (Fig. 5.21 and Table 5.9). R values appeared to highest in colonies from the northern subpopulation and the single colony in Limerick (colony 3), and lowest in the colonies in Clare and south Galway. However, the higher level of relatedness seen in the northern colonies could be partly due to the lack of heterozygosity at one locus (RHC108b) in this subpopulation. This locus was monomorphic in this subpopulation, with the exception of a single heterozygote from the Ross House colony. Sites where the mean relatedness (± SD) fell within the upper and lower confidence intervals around zero indicate that the sampled individuals at these sites showed no relatedness; only one colony (Ballyallaban, colony 10) showed no relatedness between the sampled individuals (Fig. 5.21 and Table 5.9), which may be partially explained by the small number of individuals samples (n = 8).

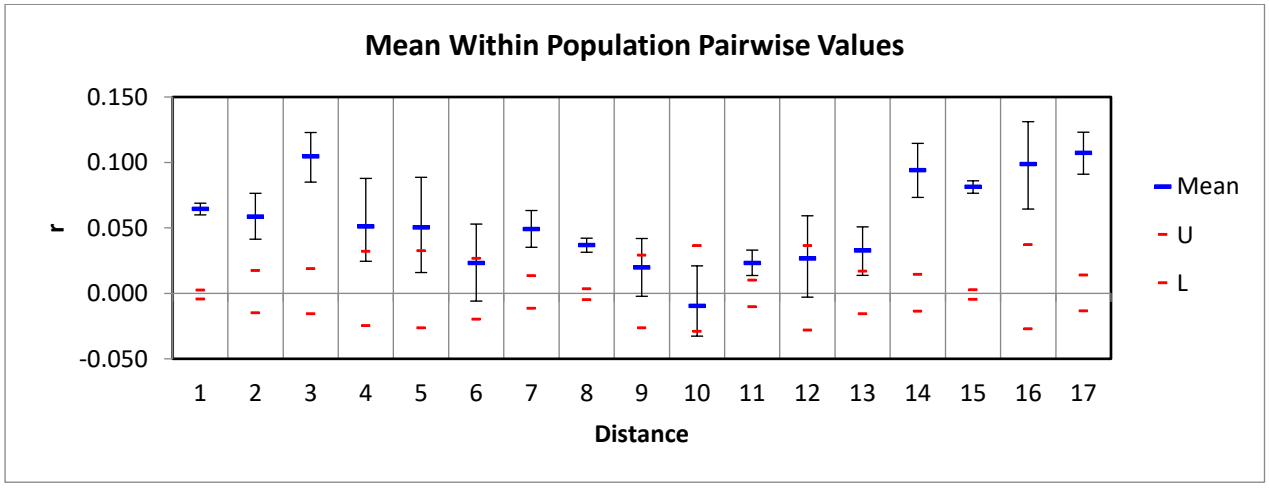

Figure 5.21: Average relatedness (r) figures for each of the 17 colonies studied, numbered as per Table 5.6, are indicated by blue box plots with standard error shown using error bars. Upper (U) and lower (L) 95% confidence limits around 0 (determined according to sample size) are shown in red, indicating sites where there is no relatedness.

Table 5.9: Table of pairwise relatedness estimates for each colony sampled. N = number of pairwise comparisons, mean = average relatedness (r) value, U = upper 95% confidence interval around 0, L = lower 95% confidence interval around 0, P(mean-rand >= mean-data) = probability for average r based on permutations across the entire data set. Mean values for colonies falling within the upper and lower confidence intervals indicate no relatedness between sampled individuals at that site.

No.	Colony	N	Mean	U	L	P(mean-rand >= mean-data)
1	Derrycreha	2016	0.064	0.002	-0.004	0.001
2	Courtney's Cottage	105	0.059	0.017	-0.005	0.001
3	Curragh Chase	105	0.105	0.019	-0.016	0.001
4	Cullaun House	36	0.051	0.032	-0.025	0.006
5	Corbally	36	0.050	0.033	-0.027	0.006
6	Knockaskibbole & Knockanean	55	0.023	0.027	-0.020	0.036
7	Rylane	190	0.049	0.013	-0.012	0.001
8	Toonagh	1378	0.037	0.003	-0.005	0.001
9	Dromore	36	0.020	0.029	-0.026	0.076
10	Ballyallaban	28	-0.009	0.036	-0.029	0.665
11	Fiddaun & Garryland	276	0.023	0.010	-0.010	0.001
12	Ballylee	28	0.026	0.036	-0.028	0.069
13	Cloghballymore	105	0.033	0.017	-0.016	0.002
14	Menlo Castle & Ross House	136	0.094	0.014	-0.014	0.001
15	Ballykine	1953	0.081	0.003	-0.005	0.001
16	Lough Mask House & Inishmaine	28	0.099	0.037	-0.027	0.001
17	Bunnadober	153	0.107	0.014	-0.013	0.001

5.4 Discussion

The various analyses performed on the microsatellite data obtained in this study showed a consistent pattern of genetic structure within the Irish lesser horseshoe bat population. STRUCTURE analysis supported a value of K = 3, which would divide the Irish population into three main geographic groups or subpopulations: southern (including colonies in Cork and Kerry), central (including Limerick, Clare and south Galway), and northern (including north Galway and Mayo). This was supported by PCoA which showed colonies from these three subpopulations clustering separately. In addition, pairwise F_{ST} values between colonies from different subpopulations were significant in almost all cases, further indicating differentiation between the subpopulations. Also, while a significant pattern of isolation by distance was found, the variance was relatively low, indicating that IBD alone did not fully explain the genetic variation seen.

In addition to the large-scale genetic structure which was observed, analysis of each subpopulation revealed evidence of possible further genetic structure on a smaller scale. Both the central and northern subpopulations showed evidence of potential weak genetic structure, based on analysis using STRUCTURE, PCoA and pairwise F_{ST} values. In these subpopulations, semi-isolated groups of colonies in Limerick and north Galway were differentiated from the other colonies in each subpopulation. However, there is also a possibility that this apparently weak structure may mask a further important population subdivision. In particular, relatively few individuals were sampled from the Limerick region, and including more individuals from this region in future analysis may show that this group of colonies is actually more distinct than was found in this study. On the other hand, it should be noted that these apparent subdivisions within the main subpopulations only became apparent when the LOCPRIOR function was used. This function is designed to assist in discerning weak population structure, but it also poses a risk of false positives, identifying structure where none may actually be present. In this case, stronger evidence for this weak population substructure may require more sampling of individuals from colonies in Limerick and North Galway. The southern subpopulation did not show any evidence of further genetic structure in this study, but this may simply be due to the small number of colonies and individuals sampled from this area in comparison to the other two subpopulations.

Despite the strong pattern of genetic structure within the population, there also appears to be evidence that interbreeding continues to occur at a low level between the three subpopulations

or did so until relatively recently, and that the three main subpopulations are not completely isolated. STRUCTURE plots for the analysis of the overall population showed individuals within each subpopulation with mixed ancestry from the three genetic clusters. The PCoA by individuals also showed overlap between individuals from the three subpopulations, and the AMOVA showed that most of the variation seen occurred within subpopulations, with only 14% of variance occurring between subpopulations. F_{IS} values and the mean relatedness of individuals for each colony were low overall, indicating that inbreeding was not occurring at these sites.

In a previous study of the population genetics of the lesser horseshoe bat in Ireland, Dool et al. (2016) came to the conclusion that the Irish population of this species was split into two main subpopulations, one in Cork and Kerry and the other consisting of all other colonies to the north. While Dool et al.'s (2016) study indicated that the most northerly colonies sampled in Mayo were also partially differentiated, this study shows more clearly that there are in fact three main lesser horseshoe bat subpopulations in Ireland. This study sampled more colonies and individuals in the northern part of the lesser horseshoe bat's range in Ireland (from Limerick northwards) in comparison to Dool et al. (2016), which more heavily sampled colonies and individuals from Cork and Kerry. In this study, 274 individuals from 18 colonies were sampled from the northern part of the lesser horseshoe bat range, in comparison to 180 individuals from 14 colonies sampled by Dool et al. (2016).

This study has shown that the lesser horseshoe bat colonies in north Galway and Mayo form a genetically distinct northern subpopulation of this species. The point separating this subpopulation from the central subpopulation in south Galway and Clare is an area to the south-east of Galway City (the "Galway Gap"). Between the closest known maternity colonies in the northern and central subpopulations (Menlo Castle and Cloghballymore, respectively), there are no known maternity or hibernation roosts for an overland distance of approximately 22 km. However, some night roosts have been recorded and lesser horseshoe bats have been radio tracked on the eastern outskirts of Galway City, 5 km from Menlo Castle (P. Scott, pers. comm.).

Lesser horseshoe bats generally travel less than 20 km from their home colony (Dietz et al., 2009). The Galway Gap is only slightly larger than this distance and contains some patches of suitable woodland habitat, meaning that lesser horseshoe bats may potentially be able to travel between the two closest colonies in the neighbouring subpopulations. However, movement for

lesser horseshoe bats may be made more difficult in this area by a dense human population. As well as the presence of Galway City and several other towns and villages, the area is crossed by several large roads, including a motorway. The combination of urbanisation and large roads may complicate the route needed to cross this area by lesser horseshoe bats, as motorways are known to pose a major barrier to movement for many bat species (Altringham and Kerth, 2016). The planned construction of a large roadway to bypass Galway City in the near future may erect a further barrier for lesser horseshoe bats attempting to travel across this area, although it should be noted that extensive survey work on the resident lesser horseshoe bat population has taken place during the planning of this project (P. Scott, pers. comm.). There may also be a shortage of suitable roost sites for lesser horseshoe bats, as many disused farm buildings in the area surrounding Galway City are known to have been converted into dwelling houses during the 1990s and 2000s (K. McAney, pers. comm.).

As recommended by Pritchard and Wen (2003) and Janes et al. (2017), further analysis of genetic clusters identified by STRUCTURE was carried out. The possible regional scale genetic structure discovered in this way in the northern and central subpopulations was supported by PCoA and pairwise F_{ST} values, although it should be noted again that the support for these clusters in STRUCTURE is very weak. These indicate that within the central subpopulation, the Limerick colony sampled in this study may be genetically distinct from the colonies in Clare and south Galway to the north. Similarly, in the northern subpopulation one of the colonies in north Galway may be distinct from the other colonies in North Galway and Mayo. Although these patterns of genetic structure appear to be weak, they do reflect the relative geographical isolation of these colonies in Limerick and north Galway.

While only a single colony (Curragh Chase) from County Limerick was sampled in this study, it is likely that it reflects the situation of all of the small number of colonies present in this area. This small cluster of colonies is separated from the nearest colonies to the north by a significant distance of at least 18 km. A large natural barrier may also be contributing to their isolation, as the Limerick colonies are separated from the Clare colonies by the Shannon estuary, a large coastal inlet.

The pattern of isolation of one of the North Galway colonies (Ross House) from all of the other colonies within the northern subpopulation is more difficult to explain. To the north, a significant distance of 18 km separates it from the nearest colony in County Mayo, and a large natural barrier is also present to the north and east in the form of Lough Corrib, the second

largest lake in Ireland. However, it is also apparently isolated from the only other colony in North Galway (Menlo Castle), although no apparent natural barrier and a shorter distance (14 km) separates them. The apparently closer relationship of the Menlo Castle colony to the colonies in South Mayo may imply that undiscovered lesser horseshoe bat colonies may be present on the eastern side of Lough Corrib, linking Menlo Castle with the other colonies to the north. However, as very few individuals were obtained from Menlo Castle, a larger number of samples is required to definitively establish the relatedness of the bats at this site with the other colonies within the northern subpopulation.

The natural barriers likely increase the minimum distance which individual bats would need to travel to the nearest colonies to facilitate gene flow, as suitable habitat and crossing points for lesser horseshoe bats may be limited. Although no conclusions could be drawn about genetic structure within the southern subpopulation, it may potentially also be subdivided into partially isolated groups due to the geographic nature of the area, which contains numerous mountain ranges and deep coastal inlets.

This study has uncovered extensive genetic structure within the Irish lesser horseshoe bat population, to an even greater extent than was previously known. This study found a similar level of genetic diversity (as measured by heterozygosity) and a low level of inbreeding across all colonies, both of which were comparable to values reported by Dool et al. (2016). However, the Irish lesser horseshoe bat has the lowest level of genetic diversity of any population of this species and is completely isolated (Dool et al., 2013; Dool et al., 2016).

The highly subdivided nature of the Irish lesser horseshoe bat population poses a threat to its long-term survival. In the absence of adequate gene flow, each subpopulation will be more prone to inbreeding depression and potential local extinction, in turn decreasing the prospects of survival for the surviving subpopulations. In the future, active conservation measures will be needed to improve connectivity or even create entirely new habitat corridors between subpopulations, in order to maintain gene flow and the genetic diversity of the Irish population as a whole.

5.5 Conclusion

- Significant genetic structure was found within the Irish lesser horseshoe bat population, with three main subpopulations identified in the southern (Cork and Kerry), central (Limerick, Clare and south Galway) and northern (north Galway and Mayo) parts of its range.
- The northern subpopulation was found to be significantly differentiated from the other subpopulations to the south, with an area in the region of Galway City apparently posing a significant barrier to movement for lesser horseshoe bats.
- Further potential weak genetic structure was identified within the northern and central subpopulations, which may be due to the presence of natural barriers and relatively large distances between groups of colonies within these subpopulations.

Chapter 6

General discussion

6.1 Development of bat species identification assays

The set of real-time PCR primers for bat species identification which was developed during this project provides a useful tool for bat surveyors and future scientific studies.

The potential of this set of assays to increase knowledge of the resident bat species in Ireland and Great Britain (and further afield) through the testing of non-invasive DNA samples such as bat droppings was shown by the large number of previously unrecorded bat roosts of seven different species discovered in Counties Waterford, Kildare, Wexford and Galway. Of particular significance were the Natterer's bat and whiskered bat roosts discovered, nearly all of which were found in County Waterford. The number of known roosts for this species in County Waterford was quadrupled by this study, from two roosts for both species (Roche et al., 2014), to eight roosts in total for Natterer's bat and nine for the whiskered bat. The newly discovered roosts also significantly increased the number of roosts known nationally for both species. The number of Natterer's bat roosts was increased by 11% from the 66 previously known across the country (Roche et al., 2014). For the whiskered bat, the number of roosts was increased by 17% from the 41 previously known (Roche et al., 2014).

As these bat species are widespread but scarce throughout Ireland and are difficult to identify to species using bat detector surveys (Roche et al., 2014), non-invasive surveys of potential roost sites for these species may provide a means of obtaining more data on their distribution. Certain parts of Ireland with suitable habitat have been highlighted by Lundy et al. (2011) as having higher potential for the presence of these species, and these areas could be selected for non-invasive roost surveys.

The usefulness of the real-time PCR primers was also shown during subsequent work on the lesser horseshoe bat. The primers specific to this species allowed DNA analysis of faecal samples to be streamlined by ranking the quality of DNA in each according to Ct value. Samples which may have come from non-target species were excluded from further analysis, and genotyping and sex typing were carried out in order of DNA quality to ensure that the success rate was as high as possible.

Although sets of species identification assays have been previously designed for British bat species, including Boston et al. (2011) and Hamilton et al. (2015), this is the first study which has created a comprehensive set of such assays for all of the resident species of the British Isles, and indeed no such set of species identification assays has been previously created for

any other region of Europe. In addition, the real-time PCR method used in this study has clear advantages over those used by Boston et al. (2011) and Hamilton et al. (2015) in terms of reduced time and cost involved in DNA analysis, its particular suitability for use with non-invasively collected DNA samples, and its potential use for down-stream genetic analysis as has been demonstrated in Chapters 4 and 5. Therefore, this set of primers will be useful for surveys and studies across the British Isles in the future, and with some modification and design of additional primer sets, it could form the basis of similar comprehensive sets of species identification assays for other regions of Europe.

6.2 Examination of the sex ratio and refining population estimates for the lesser horseshoe bat in Ireland

This project successfully determined the sex ratio of adult lesser horseshoe bats at six summer roosts across this species' range in Ireland. The proportion of males at these sites was found to be highly variable, from 14.2% (±5.6%) to 74.3% (±7.1%). The overall estimated mean proportion of males ranged from 47.1-56.8%. This contrasts greatly with previous studies, such as the Welsh roost studied by Bontadina et al. (2002) where 24.4% of individuals were male, and Zarzoso-Lacoste et al.'s (2018) study in France, where the proportion of males in lesser horseshoe bats in 19 summer roosts prior to the birth of pups ranged from 0% to 50%, and the overall proportion of males was 25.8%.

The mean proportion of males found in this study is also higher than the 25% mean proportion of males in Irish summer roosts which is used in calculations of Ireland's national population of this species (Roche et al., 2012; NPWS, 2013). The results of this study imply that the Irish lesser horseshoe bat population actually ranges from 9,340-9,882 individuals, which is 29.5-33.3% smaller than the previous estimate of 14,010 individuals. While this significant downwards revision of the population estimate is a cause for concern, it should be remembered that the Irish population has gradually increased by between 60-97% since 1993 (Roche et al., 2014), and a more accurate population estimate will allow more informed conservation management decisions to be taken in the future.

It is unclear why the proportion of male lesser horseshoe bats in summer roosts is so high in Ireland in comparison with France, with males making up a large majority of the biggest colony that was sampled, at Courtney's Cottage. This could be explained by a shortage of suitable roosts for this species in Ireland, as Roche et al. (2015) have shown the state of repair of many roost structures used by lesser horseshoe bats is poor and deteriorating steadily, causing

population declines of many individual colonies. Therefore, males may be forced to roost in the same sites as breeding females in Ireland, when they would not otherwise do so in other countries. Interestingly, the largest proportion of males was discovered at the two roosts with the largest populations (Garryland and Courtney's Cottage), both of which have been extensively repaired and remodelled to improve conditions for the bats (pers. obs.). This raises the possibility that male bats are actively migrating to roost sites which provide better conditions than other suboptimal sites, as they are thought to be less strongly philopatric than female lesser horseshoe bats (Gaisler, 1963; Dool et al., 2016). On the other hand, the site with the lowest proportion of males (Curragh Chase) has also been modified to improve conditions for the resident bats (pers. obs.), but this possibility should be investigated further by examining the sex ratio of bats at other roosts which have been extensively restored.

Another potential factor contributing to the higher proportion of males observed in lesser horseshoe bat roosts in Ireland may be climatic conditions. The lesser horseshoe bat has been classed as belonging to a "Mediterranean" biogeographic group of European bats by Rebelo et al. (2010) based on the climatic conditions at the sites it is known to occupy throughout Europe. Gaisler (1963) noted that lesser horseshoe bats in Czechoslovakia tended to occupy different parts of their summer roosts in buildings to take advantage of differing microclimates depending on their sex, with the majority of adult females and small numbers of males tending to roost in large clusters in warm areas such as attic spaces, while most males and some non-breeding females tended to be scattered throughout cooler parts of the building. While this has been assumed to be the general pattern of behaviour of lesser horseshoe bats throughout their range, it could be expected that the relatively cool average temperature in Ireland may cause it to modify its behaviour in comparison to areas which are more climatically favourable.

Schofield (1996) found that the optimum ambient air temperature for lesser horseshoe bats to minimise their energy expenditure in keeping warm is 30 °C. It was also discovered that the formation of clusters of lesser horseshoe bats within their roosts during the daytime could create a microclimate of 30 °C, which was an average of 14.6 °C warmer than the ambient air temperatures within the roosts studied. Although Schofield (1996) did not examine the sex ratio of the bats present in these roosting clusters, it is possible that male lesser horseshoe bats in Ireland may take advantage of the roosts with a warmer microclimate which are usually mainly preferred by females in other regions, as ambient air temperatures here rarely reach the optimum 30 °C.

While this study has obtained the first data on the sex ratio of bats at lesser horseshoe bat summer roosts in Ireland, a relatively small proportion of the summer roosts which are regularly surveyed as part of the national monitoring scheme has been sampled, less than 5%. As the sex ratio appears to vary so widely between colonies, additional sites should be studied in the future to increase the sample size. Surveying at least a further six summer roosts would provide a minimum sample of approximately 10% of the monitored roosts, and sex typing 196 faecal DNA samples from each of these would provide a sex ratio with a maximum error of ±7%. Additional sampled roosts should include varying colony sizes, geographical locations and states of repair to account for the possibility of widely varying sex ratios depending on these variables. In addition, a subset of roosts should be sampled several times in a summer season prior to the time that new pups begin to fly, as the sex ratio of adult bats at colonies may potentially vary over time, as shown by Zarzoso-Lacoste et al. (2018).

Overall, this study is highly novel in several respects. Firstly, it is (to the best of the author's knowledge) one of only three studies worldwide which have applied molecular methods to the sexing of bat species, along with Korstian et al.'s (2013) study of a set of North American bat species and Zarzoso-Lacoste et al.'s (2018) study of *R. hipposideros* in France. The lack of studies using such molecular sex typing methods for bat species appears to be at least partly a result of the continued use of invasive sampling of bat species by researchers (pers. obs.), whereby the need for molecular sex typing methods are avoided by direct sexing of trapped bats during sampling. However, the potential advantages of using novel molecular sex typing methods in tandem with non-invasive sampling are clearly illustrated by the insights of this study and Zarzoso-Lacoste et al. (2018) into the social structure of *R. hipposideros*, which simply could not have been obtained by using the invasive methods more commonly used by bat researchers. Secondly, this study is also unique in using real-time PCR technology for molecular sex typing, which has several advantages over the conventional PCR methods used by the other two studies in terms of sensitivity, streamlining of laboratory analysis, and the potential for screening of samples for further downstream analysis (O'Neill et al., 2013). For these reasons, the primer sets designed in this study for molecular sex typing of *R. hipposideros* are potentially more useful than those published by Zarzoso-Lacoste et al. (2018).

In addition to its use of novel technology in the context of the study of bat populations, it is only the second study to examine the social structure of *R. hipposideros* colonies in the context of the sex ratio of adult bats present in summer roosts, which is of importance in obtaining accurate population estimates of this species to inform conservation decisions. The large

difference seen in the mean sex ratio of bats in *R. hipposideros* colonies between this study and Zarzoso-Lacoste et al. (2018) highlights the importance of sampling in different parts of a species' range, as data gathered from populations occupying regions of optimal habitat (as in Zarzoso-Lacoste et al., 2018) may not accurately reflect the situation in populations occupying possibly suboptimal, edge-of-range regions like Ireland.

As well as having the potential to be used for further studies to examine the social structure of *R. hipposideros* in other European populations, the real-time PCR sex typing primers designed in this study may also provide the basis for designing similar assays for other closely related *Rhinolophus* bat species, of which there are four present in Europe: *R. ferrumequinum*, *R. euryale*, *R. mehelyi* and *R. blasii* (Dietz et al., 2009). *R. euryale* has an IUCN conservation assessment of Near Threatened and *R. mehelyi* is Vulnerable, while the other two species are of Least Concern, but the populations of all four species are believed to be decreasing (Alcaldé et al., 2016; Juste and Alcaldé, 2016; Piraccini, 2016; Taylor, 2016b).

All of these species are thought to have broadly similar social systems to *R. hipposideros*, with large summer colonies of sometimes several thousand individuals consisting mainly of breeding females, but with a significant proportion of adult males (Dietz et al., 2009). However, there is very little data on the sex ratio of these bat species at their summer roosts. For *R. ferrumequinum*, an estimated 25% of bats in summer roosts in England are males, but possibly less than 1% of bats in roosts in the Mediterranean region are males, while for *R. mehelyi* and *R. blasii*, up to one third of bats in summer roosts can consist of males (Dietz et al., 2009). Therefore, the same research question posed in this study has relevance for these species, and is of significance in helping to obtain accurate population estimates in the future. Puechmaille and Teeling (2014) demonstrated the use of non-invasive genetic sampling for species identification of *R. mehelyi* and *R. euryale* in cave roosts in France, showing that this approach could also be successful in the future for the investigation of population sex ratios using molecular methods. While it was not tested in this study, it is possible that the sex typing primers designed in this study may be directly applied to use for these species due to their close relationship to *R. hipposideros*, or may at least be redesigned for this purpose. In a wider context, the question in this study and the non-invasive approach and molecular methods used for sex typing also have relevance for all other European bat species, and could be applied more widely beyond *Rhinolophus* species.

6.3 Population genetics of the lesser horseshoe bat in Ireland

This project also examined the population genetics of the Irish lesser horseshoe bat population, and found that it contains more genetic structure than previously thought, indicating the potential presence of several important barriers to the movement of this species across its range. The population is split into three main subpopulations, two of which (the northern and central subpopulations) also appear to be possibly further weakly subdivided. There is no evidence that the southern subpopulation is similarly subdivided, but this study did not have enough data to examine this properly. This picture contrasts with Dool et al.'s (2016) study which found that only two main lesser horseshoe bat subpopulations exist in Ireland, but the difference may lie in the more extensive sampling in the northern part (from Limerick northwards) of the lesser horseshoe bat's range by this study.

As the Irish lesser horseshoe bat population is completely isolated from those in the rest of Europe (Dool et al., 2016), this level of genetic structuring poses a serious risk to the future survival of this population. Lesser horseshoe bats in Ireland essentially appear to inhabit a series of population "islands" which are blocked from interbreeding to varying extents. Although inbreeding was not excessive at any of the colonies sampled and there appears to be a low level of interbreeding between the three subpopulations, these largely isolated groups may be at risk of inbreeding depression or local extinction in the long term if the isolation continues (Dool et al., 2016).

For each of the five subpopulations identified across the species' range, one or more of several factors may be contributing to isolation. These include the presence of natural barriers, the presence of large cities and towns in certain areas, a lack of suitable roosting sites, a lack of suitable habitat or habitat fragmentation, and simple geographic distance. Isolation by distance has been shown to occur across the horseshoe bat's range in Ireland in this study and by Dool et al. (2016), which could be expected for a bat species which is generally thought to be highly sedentary and faithful to its roost sites. However, although some of the other factors may appear to be obviously affecting the movement of horseshoe bats (e.g. the presence of the Shannon estuary or Galway City between two of the subpopulations identified), there is little or no evidence to support this, apart from some anecdotal reports. In particular, although the lack of suitable habitat could be expected to influence the distribution of this species, Dool et al. (2016) found that habitat suitability was not responsible for the genetic variability seen, which was unexpected. However, it is possible that the geographic scale at which the analysis was carried

out was not fine enough to assess the true importance of small-scale habitat features in the landscape. Therefore, further research is needed to definitively establish the reasons for the presence of these barriers between each horseshoe bat subpopulation.

Conversely, the apparent lack of strong genetic structure between apparently geographically isolated groups of colonies may indicate the presence of as yet undiscovered lesser horseshoe bat colonies providing a connection between them. In particular, the County Limerick group of colonies appears not to be highly genetically distinct from the colonies in County Clare, despite being separated by the Shannon estuary and Limerick City, and a relatively long distance (at least 18 km). This suggests that undiscovered lesser horseshoe bat colonies may exist somewhere in between these groups of colonies, allowing gene flow to continue and preventing stronger genetic structure from developing. If such important "bridge" colonies do exist, survey work to identify them is necessary in the near future to ensure their protection, although it should be noted that extensive searches for lesser horseshoe bat colonies have already been carried out in some of these areas (McAney et al., 2013).

In the long term, the highly subdivided nature of the Irish lesser horseshoe bat population poses a threat to its continued survival, due to the risk of the development of inbreeding depression, and the possibility of local extinctions of small populations due to random stochastic events (Lande, 1993). Human activity has had a major impact on the distribution, ecology and population genetics of the lesser horseshoe bat in Ireland for at least the past few centuries, and continues to do so at present (Dool et al., 2016). If it is to survive into the future, human intervention of some kind will be required.

Conservation management of this species thus far has focused on protecting the currently known colonies of this species, and conserving certain areas of habitat within its range as Special Areas of Conservation under the EU Habitats Directive, and this has successfully allowed the population to increase significantly over the last three decades from a very low level (Roche et al., 2014). However, a more active and ambitious conservation management plan will be needed for the coming decades, as much of its roosting resource, even in core parts of its range, is declining in quality (Roche et al., 2015) and it is failing to recolonise areas which would reconnect the existing subpopulations. In order to facilitate population expansion, recolonisation of new areas and reconnection of the current subpopulations, actions will need to be taken at a landscape level, including the restoration of buildings which currently contain

colonies, the creation of a new bank of potential roost sites, and the restoration of large areas of suitable habitat for foraging and long-distance movement (McAney, 2014).

The roosting preferences of lesser horseshoe bats in Ireland have been well studied, favouring a wide variety of old buildings in rural areas (Roche et al., 2014). A large number of old farm buildings, dwelling houses, manor houses and other similar buildings exist across Ireland which have the potential to be used by this species, but many have fallen into serious disrepair or dereliction due to changes in farming practices and other social changes (Heritage Council, 2005). Many of these buildings have value as part of the heritage and history of Ireland, and some schemes exist to provide government funding towards their restoration and maintenance, such as the Heritage Council's Traditional Farm Buildings Grant Scheme. Although these schemes do not currently specify that the buildings should include a benefit to wildlife, some of these buildings in appropriate areas could be restored partially with the intention of housing of lesser horseshoe bat colonies. The restoration of existing roost buildings by the Vincent Wildlife Trust and the National Parks and Wildlife Service has resulted in population increases at a number of lesser horseshoe bat colonies (Roche et al., 2014; VWT, 2018). Three newly built underground hibernation roosts built by the NPWS in recent years have also been occupied by lesser horseshoe bats in the space of several years (R. Stephens, pers. comm.). This demonstrates the ability of lesser horseshoe bats to colonise new roosts where these are located sufficiently close to existing roosts, and that a programme of creation of new roost sites in specific areas could lead to a gradual expansion of this species' range.

As well as an increase in the number of potential roost sites, the future expansion of the lesser horseshoe bat's range will also likely require an increase in the amount of suitable habitat for foraging and long distance movements. Ireland has the lowest proportion of woodland cover of any country in Europe, approximately 11.5% (Eurostat, 2013). Only about 2% of land cover consists of broadleaf and mixed woodland (Perrin et al., 2008), which is the preferred foraging habitat of the lesser horseshoe bat, with the remainder made up of non-native conifer plantations which are largely managed under a system of periodic clear-felling. Large-scale woodland habitat restoration may be difficult to achieve in Ireland due to the presence of a large number of relatively small land holdings, but gradual change in the coming decades may be possible with government support. The Irish government plans to increase the proportion of broadleaved woodland in Ireland to 30% of new forestry planted, as well as increasing overall woodland to 18% of land cover by 2046 (DAFM, 2015). A targeted effort to increase the amount of broadleaf woodland habitat in certain areas may encourage recolonisation by lesser

horseshoe bats by providing increased foraging habitat, but this is likely to take several decades at least.

In the interim, corridors of woodland habitat could be created to allow lesser horseshoe bats to migrate more easily between the present subpopulations and maintain gene flow. Potential long-distance woodland corridors could be created along former railways, many of which have been reopened for public use as "greenways", as well as still-functional railways. Riparian woodland corridors could also be planted along river valleys, as this is a habitat known to be favoured by lesser horseshoe bats (Roche et al., 2014). In the case of the cities of Galway and Limerick, their development plans and biodiversity action plans, which are periodically reviewed, could also provide for the creation of corridors of natural habitats including woodland. In addition to allowing for the migration of lesser horseshoe bats and other species, they would also have amenity value for local people.

This project has highlighted the vulnerability of the lesser horseshoe bat in Ireland, with a significantly smaller and more divided population than previously thought. Although it has a high level of protection and its population continues to grow, its future is uncertain. Major measures will need to be applied on a landscape scale to ensure its survival in Ireland, some of which may take decades to implement. Therefore, a long-term action plan for the conservation of the lesser horseshoe bat is needed in the near future.

The results of this study have the most relevance for the conservation of the lesser horseshoe bat in Ireland, but they also have a wider significance internationally. Both this study and Dool et al. (2016) have shown that the Irish lesser horseshoe bat population is subdivided into smaller subpopulations, a situation which potentially threatens its long-term future. While it is a relatively small population, lesser horseshoe bat populations in many countries in western and central Europe declined massively during the 20th Century (Dietz et al., 2009), so it is still worthy of conservation and is important for the future of the species within Europe. No similar national scale population genetics study has been carried out on the lesser horseshoe bat in Britain (Dool et al. 2016), or elsewhere in Europe. Given the serious contraction in the species' range and decline in numbers in the past 70 years, an examination of the population genetics and extent of gene flow among colonies within the lesser horseshoe bat's range in western and central Europe should be prioritised, as the example of Ireland's population shows that unsuspected population subdivisions and barriers to gene flow could pose a risk of local extinctions of isolated subpopulations.

In addition, although several studies have demonstrated the potential usefulness of non-invasively collected faecal DNA samples for population genetics studies of bat species (Puechmaille et al., 2005; Puechmaille et al., 2007; Puechmaille et al., 2007; Boston et al., 2012), or have used a small number of faecal DNA samples in larger bat population genetics or phylogenetics studies mainly relying on tissue samples (Goodman et al., 2012; Ruedi et al., 2012; Dool et al., 2013), this is, to the author's knowledge, the first study worldwide to solely use non-invasively collected faecal DNA samples to examine the population genetics of a bat species on a national scale. The success of this study in doing so demonstrates the usefulness of this method, and the potential for the non-invasive approach to be applied more widely in population genetics studies on other bat species worldwide in the future. It has also shown how the real-time PCR species identification assays described in Chapter 2 can be used for screening of faecal DNA samples for selection of the highest quality samples, which will further improve the efficiency of such studies.

6.4 Future work

- Non-invasive surveys of potential roost sites for Natterer's bat and whiskered bat could be carried out across Ireland, using the real-time PCR species identification primers designed in this project. Such surveys could be targeted towards parts of the country which have been predicted to have high potential for the presence of these species.
- Genotyping and sex typing should be completed for DNA samples obtained during this project in the lesser horseshoe bat roosts at Curragh Chase, Garryland and Courtney's Cottage. This would give an accurate estimate of the sex ratio from all six roosts which were sampled and would provide further data to improve the national population estimate for this species.
- Analysis of the sex ratio of adult lesser horseshoe bats should be carried out at a number of other summer colonies in Ireland, as sex ratios may vary depending on the size of the colony, the state of repair of the roost building, or other factors. Sex typing of 196 DNA samples each from another six colonies of varying population sizes would give information on 10% of the known summer colonies. Sex ratios should also be examined at a number of roosts several times over the course of a single summer season as this may also vary.
- Sampling of a larger number of individual genotypes from more colonies to add to the present population genetics analysis could provide more information on the pattern

already seen. In particular, more colonies and/or individuals could be sampled from the Limerick population, and Menlo Castle-Ross House, to investigate whether the weak structure already seen resolves into higher scale structure, as a relatively small number of individuals were sampled from these areas. Genotypes are also lacking from the most northerly lesser horseshoe bat colonies in Co. Mayo, which are separated from the main cluster sampled in this study by about 15 km and another natural barrier (Lough Carra). Regional genetic structure within the southern subpopulation in Cork and Kerry should also be investigated, as the potential is high given the number of mountain ranges, large lakes and deep coastal inlets to present potential barriers to movement.

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Appendix 1: Concentration and purity of DNA extracted from tissue samples.

Sample no.	Extract	Species	Concentration (ng/µl)	260/280
1270	AH150313.10	*P. pygmaeus*	131.8	1.84
3016	AH150313.11	*P. pygmaeus*	96.3	1.88
2956	AH150313.12	*P. pygmaeus*	96.4	1.85
2800	AH150313.13	*P. pygmaeus*	28.8	1.92
2797	AH150313.14	*P. pygmaeus*	39.6	1.91
2799	AH150313.15	*P. pygmaeus*	87.8	1.83
2962	AH150313.16	*P. pygmaeus*	77.1	1.85
2798	AH150313.17	*P. pygmaeus*	123.2	1.88
4844	AH210613.2	*P. pygmaeus*	92.6	1.86
3132	AH210613.1	*P. pygmaeus*	62.5	1.86
1269	AH210613.3	*P. pygmaeus*	210	1.82
1230	AH021013.1	*M. mystacinus*	16.2	1.52
645	AH021013.2	*P. pygmaeus*	41	1.74
646	AH021013.3	*P. pipistrellus*	153.2	1.8
647	AH021013.4	*P. auritus*	280.5	1.85
648	AH021013.5	*P. auritus*	29.5	1.59
14032	AH011114.1	*M. nattereri*	66.4	1.79
14034	AH011114.3	*N. leisleri*	7.8	1.75
14036	AH011114.5	*R. hipposideros*	6.6	1.45
14037	AH011114.6	*P. pygmaeus*	6.1	2.33
14041	AH291114.1	*P. pipistrellus*	4.1	1.5
14042	AH291114.2	*P. pygmaeus*	32.8	1.8
14043	AH291114.3	*P. auritus*	1.1	1.83
14044	AH291114.4	*P. pygmaeus*	13.2	1.73
14045	AH291114.5	*P. nathusii*	6.3	2.1
14651	AH160615.1	*M. daubentonii*	106.4	1.85
BECH1	AH160615.2	*M. bechsteinii*	5.4	1.82
BECH2	AH160615.3	*M. bechsteinii*	2.7	0.92
BECH3	AH160615.4	*M. bechsteinii*	9.8	1.55
BECH4	AH160615.5	*M. bechsteinii*	4.5	1.44
14656	AH160615.6	*R. hipposideros*	5.8	1.68
14689	AH160615.7	*R. hipposideros*	6.7	1.72
14690	AH160615.8	*R. hipposideros*	8.2	1.77
14691	AH160615.9	*R. hipposideros*	18.6	1.65
14692	AH160615.10	*R. hipposideros*	21.4	1.74
JP2		*M. myotis*		

Appendix 2: Details for Cyt b gene sequences downloaded from Genbank for primer design.

Barbastella barbastellus					
JQ683176	JQ683183	JQ683190	JQ683197	JQ683204	JQ683211
JQ683177	JQ683184	JQ683191	JQ683198	JQ683205	JQ683212
JQ683178	JQ683185	JQ683192	JQ683199	JQ683206	
JQ683179	JQ683186	JQ683193	JQ683200	JQ683207	
JQ683180	JQ683187	JQ683194	JQ683201	JQ683208	
JQ683181	JQ683188	JQ683195	JQ683202	JQ683209	
JQ683182	JQ683189	JQ683196	JQ683203	JQ683210	
Eptesicus serotinus					
KF019046	KF019047	KF019048	KF019049	KF019063	KF019064
Myotis alcathoe					
DQ120882	EU541661	EU541663	EU795690		
DQ120883	EU541662	EU541664	EU795691		
Myotis bechsteinii					
AF376843	DQ120899	DQ120900	DQ120901		
Myotis brandtii					
AF376844	AM261886	AY665139	AY665168		
Myotis daubentonii					
AF376847	EU153102	EU153107	EU153112	EU153117	EU153122
AF376862	EU153103	EU153108	EU153113	EU153118	EU153123
DQ120896	EU153104	EU153109	EU153114	EU153119	
DQ120897	EU153105	EU153110	EU153115	EU153120	
DQ120898	EU153106	EU153111	EU153116	EU153121	
Myotis myotis					
AF246241	AF246244	AF376860	JX442102	JX442113	
AF246242	AF246245	AM261883	JX442103	JX442114	
AF246243	AF246246	JX442099	JX442105		
Myotis mystacinus					
AF376861	AY665141	DQ120880	EU360643		
AY665140	DQ120879	DQ120881			
M. nattereri					
AF376863	JN591509	JX826351	JX826357	JX826363	JX826369
JN591504	JN591510	JX826352	JX826358	JX826364	JX826370
JN591505	JX826347	JX826353	JX826359	JX826365	JX826371
JN591506	JX826348	JX826354	JX826360	JX826366	JX826372
JN591507	JX826349	JX826355	JX826361	JX826367	
JN591508	JX826350	JX826356	JX826362	JX826368	
Nyctalus leisleri					
DQ120875	DQ887592	DQ887595	EU360683	EU360686	EU360689
DQ120876	DQ887593	EU360681	EU360684	EU360687	EU360690
DQ120877	DQ887594	EU360682	EU360685	EU360688	
Nyctalus noctula					
DQ120872	DQ120873	DQ120874	JX570902		

Pipistrellus nathusii					
AH006590	AJ504446	DQ120849	DQ120850	U95509	U95510
Pipistrellus pipistrellus					
AY316338	AY316343	AY316348	AY426100	AY582290	AY663799
AY316339	AY316344	AY316349	AY582286	AY582291	AY663800
AY316340	AY316345	AY316350	AY582287	AY582292	AY663801
AY316341	AY316346	AY426098	AY582288	AY582293	
AY316342	AY316347	AY426099	AY582289	AY582294	
Pipistrellus pygmaeus					
AY316319	AY316324	AY316329	AY426088	AY582281	DQ120855
AY316320	AY316325	AY316330	AY582277	AY582282	DQ120856
AY316321	AY316326	AY316331	AY582278	AY663796	EU084882
AY316322	AY316327	AY426086	AY582279	AY663797	EU084883
AY316323	AY316328	AY426087	AY582280	AY663798	EU084884
P. auritus					
AB085734	AF513758	AF513761	AF513765	AF513769	AY665169
AF513756	AF513759	AF513762	AF513767	AJ431650	
AF513757	AF513760	AF513764	AF513768	AY306211	
P. austriacus					
KF358491	KF358497	KF358503	KF358509	KF358515	KF358521
KF358492	KF358498	KF358504	KF358510	KF358516	KF358522
KF358493	KF358499	KF358505	KF358511	KF358517	
KF358494	KF358500	KF358506	KF358512	KF358518	
KF358495	KF358501	KF358507	KF358513	KF358519	
KF358496	KF358502	KF358508	KF358514	KF358520	
Rhinolophus ferrumequinum					
DQ120919	EU360626	EU360628	EU360626		
DQ120920	EU360627	EU360629			
Rhinolophus hipposideros					
KC978518	KC978529	KC978586	KC978600	KC978652	KC978700
KC978519	KC978563	KC978587	KC978644	KC978655	KC978704
KC978520	KC978568	KC978592	KC978645	KC978666	KC978705
KC978522	KC978574	KC978596	KC978647	KC978673	KC978713
KC978528	KC978577	KC978598	KC978650	KC978699	

Appendix 3: Real-time PCR Ct values for reference samples. Ct values are listed for real-time PCR primer sets according to target species, coded as follows: *Barbastella barbastellus*- Bbar, *Eptesicus serotinus*- Eser, *Myotis alcathoe*- Malc, *M. bechsteinii*- Mbec, *M. brandtii*- Mbra, *M. daubentonii*- Mdau, *M. myotis*- Mmyo, *M. mystacinus*- Mmys, *M. nattereri*- Mnat, *Nyctalus leisleri*- Nlei, *N. noctula*- Nnoc, *Pipistrellus nathusii*- Pnat, *P. pipistrellus*- Ppip, *P. pygmaeus*- Ppyg, *Plecotus auritus*- Paur, *Pl. austriacus*- Paus, *R. ferrumequinum*- Rfer, *Rhinolophus hipposideros*- Rhip. Species assignment is indicated by Ct value in bold, underlined. DNA sample types are abbreviated as T (tissue) or F (faecal). Negative amplification is indicated by "U" (standing for "undetermined").

Extract code	**Type**	**Species**	**Bbar**	**Eser**	**Malc**	**Mbec**	**Mbra**	**Mdau**	**Mmyo**	**Mmys**	**Mnat**	**Nlei**	**Nnoc**	**Pnat**	**Ppip**	**Ppyg**	**Paur**	**Paus**	**Rfer**	**Rhip**
AH160615.2	T	Mbec	U	29.41	U	**15.39**	37.19	U	U	U	35.84	23.95	36.23	U	34.3	33.94	U	U	U	U
AH160615.3	T	Mbec	U	28.37	U	**20.72**	38.4	U	U	U	37.03	30.59	U	37.41	32.24	37.56	32.59	U	U	35.75
AH160615.4	T	Mbec	U	31.86	33.62	**17.26**	35.95	U	U	U	U	28.05	35.33	U	33.36	31.27	U	U	U	31.57
AH160615.5	T	Mbec	U	30.57	35.17	**15.73**	33.27	U	U	39.82	U	23.33	35.74	38.06	34.1	31.04	32.19	U	U	34.01
AH160615.1	T	Mdau	U	28.11	29.2	34.69	U	**15.44**	U	U	U	U	21.74	U	35.87	31.79	U	U	U	32.31
JP2	T	Mmyo	U	29.54	U	37.68	-	-	**24.01**	-	-	-	37.03	-	-	-	-	U	-	-
AH021013.1	T	Mmys	U	37.74	U	U	U	U	U	**14.14**	U	U	U	U	32.91	31.58	U	U	U	37.47
AH011114.1	T	Mnat	U	U	U	U	U	U	U	32.44	**15.11**	U	U	U	38.49	U	U	U	U	32.85
AH011114.3	T	Nlei	U	31	U	U	U	U	U	U	U	**17.02**	U	U	34.47	34.07	U	U	U	31.05
AH291114.5	T	Pnat	U	28.43	U	39.95	37.09	U	37.35	U	U	30.32	35.14	**15.2**	30.39	30.37	U	U	U	33.77
AH021013.3	T	Ppip	U	26.4	U	33.14	U	U	26.48	U	U	U	U	U	**16.11**	30.26	31.61	U	U	U
AH011114.2	T	Ppip	U	30.6	U	U	U	U	U	37.41	U	U	U	U	**17.14**	U	U	U	U	U
AH150313.10	T	Ppyg	U	32.44	U	U	38.1	U	U	36.72	U	U	35.22	27.35	29.96	**17.08**	32.86	U	U	33.29
AH150313.11	T	Ppyg	U	36.9	U	31.97	U	U	U	36.59	U	U	34	27.2	29.77	**18.18**	31.24	U	U	U

Extract code	Type	Species	Bbar	Eser	Malc	Mbec	Mbra	Mdau	Mmyo	Mmys	Mnat	Nlei	Nnoc	Pnat	Ppip	Ppyg	Paur	Paus	Rfer	Rhip
AH150313.12	T	Ppyg	U	35.69	U	U	U	U	U	36.18	U	33.85	U	26.54	35.03	**22.9**	31.01	U	U	32.33
AH150313.13	T	Ppyg	U	35.06	U	U	U	U	U	U	U	U	U	22.25	34.74	**19.4**	U	U	U	33.51
AH150313.14	T	Ppyg	U	U	U	U	U	U	U	36.15	35.05	33.92	34.99	25.28	35.29	**17.05**	U	U	U	U
AH150313.15	T	Ppyg	U	33.26	U	U	U	U	U	35.61	U	U	U	25.34	U	**16.41**	U	U	U	U
AH150313.16	T	Ppyg	U	31.68	U	U	U	U	U	36.18	U	U	U	25.46	32.48	**16.55**	U	U	U	32.3
AH150313.17	T	Ppyg	U	39.83	32.66	U	U	U	35.7	U	U	U	U	25.25	31.44	**15.7**	33.07	U	U	33.77
AH210613.2	T	Ppyg	U	26.38	U	U	U	U	33.2	37.66	U	33.21	U	25.4	32.24	**18.06**	U	U	U	33
AH210613.1	T	Ppyg	U	30.18	U	36.76	U	U	37.39	35	U	U	36.41	22.01	33.21	**15.42**	U	U	U	U
AH210613.3	T	Ppyg	33.68	24.62	U	31.56	U	U	26.62	35.03	U	U	36.33	27.96	33.78	**16.81**	U	U	U	32.16
AH021013.2	T	Ppyg	U	25.42	U	30.94	U	U	U	36.13	U	U	39.25	26.58	28.29	**18.42**	U	U	U	36.04
AH011114.4	T	Ppyg	U	U	U	U	U	U	U	37.03	U	U	U	U	30.88	**17.48**	U	U	U	U
AH291114.4	T	Ppyg	U	30.56	U	35.56	38.46	U	U	34.86	U	U	32.9	24.32	32.52	**14.62**	32.25	U	U	35.37
AH011114.6	T	Ppyg	U	U	U	39.77	37.73	33.3	U	30.12	U	U	U	26.14	27.42	**15.66**	37.34	U	U	32.6
AH021013.4	T	Paur	26.17	28.21	28	30.21	U	U	29.37	U	U	38.23	34.69	38.42	30.26	31.95	**16.54**	34.23	U	U
AH021013.5	T	Paur	32.99	32.91	U	39.23	U	U	U	U	U	U	U	U	30.4	28.41	**27.45**	U	U	U
AH291114.3	T	Paur	U	39.36	U	U	U	U	U	U	U	U	U	U	U	34.44	**15.41**	U	U	U

Extract code	Type	Species	Bbar	Eser	Malc	Mbec	Mbra	Mdau	Mmyo	Mmys	Mnat	Nlei	Nnoc	Pnat	Ppip	Ppyg	Paur	Paus	Rfer	Rhip
AH011114.5	T	Rhip	U	-	U	U	U	U	U	U	-	U	U	U	28.81	28.5	U	U	U	**15.82**
AH160615.6	T	Rhip	U	30.13	U	U	U	U	U	U	U	U	U	U	34.07	30.05	U	U	33.19	**14.92**
AH160615.7	T	Rhip	36.62	29.66	U	36.53	U	38.61	U	39.15	U	U	U	34.35	33.06	31.28	29.41	U	33.3	**15.27**
AH160615.8	T	Rhip	U	29.94	U	38.06	U	U	U	35.22	U	U	U	U	U	38.18	U	U	U	**14.42**
AH160615.9	T	Rhip	U	28.73	U	U	U	U	U	U	U	U	U	U	U	35.23	U	U	U	**14.33**
AH160615.10	T	Rhip	U	29.15	U	36.06	34.34	U	U	39.02	U	U	34.58	33.72	39.53	33.51	U	U	U	**14.23**
COR201112 4	F	Bbar	**20.29**	U	U	U	U	U	29.64	U	U	U	U	38.49	30.03	30.76	U	U	U	33.66
COR300712 6	F	Bbar	**18.54**	29.16	U	31.43	U	32.03	32.55	34.36	U	U	U	35.85	29.54	35.65	U	U	U	32.41
COR021113.1	F	Bbar	**25.01**	U	U	U	U	U	U	U	U	U	U	U	U	32.01	U	U	U	U
COR210410.7	F	Eser	U	**20.14**	U	U	U	U	30.26	U	U	U	28.86	U	37.11	U	U	U	U	U
COR180810.1	F	Eser	U	**23.24**	U	U	U	U	32.01	U	U	U	30.66	U	U	29.48	32.39	U	U	U
COR240810.4	F	Eser	U	**24.24**	U	U	U	U	30.62	U	U	U	32.96	U	35.14	U	U	U	U	U
COR270810.2	F	Eser	U	**21.25**	U	U	U	U	38.74	U	U	U	29.9	U	29.24	U	U	U	U	U
COR190511 8	F	Malc	33.12	U	**15.39**	38.59	U	20.8	31.84	21.35	U	U	34.52	37.51	31.2	33.25	U	U	U	U
COR190511.17	F	Malc	U	U	**17.91**	U	U	23.17	33.63	27.34	U	U	U	38.17	28.46	32.26	U	U	U	38.84
SGD140714 2	F	Mbra	U	29.73	U	32.12	**21.01**	U	U	37.68	U	U	U	38.69	29.83	36.67	U	U	U	38.96
ES29071310	F	Mbra	U	28.31	U	31.28	**17.07**	34.1	29.63	37.06	U	U	U	35.18	26.39	29.54	U	U	U	31.48

Extract code	Type	Species	Bbar	Eser	Malc	Mbec	Mbra	Mdau	Mmyo	Mmys	Mnat	Nlei	Nnoc	Pnat	Ppip	Ppyg	Paur	Paus	Rfer	Rhip
COR100913.1	F	Mbra	U	34.43	U	U	**26.66**	U	U	U	U	U	U	U	U	29.49	U	U	U	U
COR260810.6	F	Mdau	U	U	U	U	U	**28.21**	U	U	U	U	U	35.36	34.3	U	U	U	U	U
COR010910.1	F	Mdau	U	37.86	U	U	U	**24.53**	36.31	32.43	U	U	34.13	39.5	37.09	33.19	U	U	U	U
SGD080714-3	F	Mmys	U	U	U	U	32.06	U	**18.76**	U	U	U	34.07	28.1	27.12	U	U	U	31.34	U
SGD080714-4	F	Mmys	U	U	U	21.44	38.4	U	**17.65**	U	U	U	U	27.59	28.29	U	U	35.99	31.67	U
SGD240414 1	F	Mmys	27.66	U	31.52	U	U	U	**22.05**	U	U	U	38.62	28.73	33.7	U	U	U	33.15	27.66
SGD080714-7	F	Mnat	33.98	U	35.96	U	U	U	U	**18.36**	U	U	37.92	27.93	27.83	U	U	33.12	U	33.98
SGD100614 1	F	Mnat	33.13	U	34.22	U	U	U	32.31	**18.77**	U	U	37.04	28.69	28.06	U	U	37.94	31.84	33.13
COR050912 3	F	Nlei	U	U	U	U	U	32.78	31.64	U	**22.39**	34.73	38.34	33	35.14	U	U	U	U	U
COR060911 8	F	Nnoc	27.87	U	35.53	U	34.77	U	33.06	U	U	**21.7**	37.88	31.39	29.24	U	U	U	U	27.87
COR121010 2	F	Nnoc	U	U	U	U	33.25	U	30.62	U	U	**25.7**	36.33	31.64	35.32	U	U	39.21	U	U
COR151113.3	F	Paur	U	30.56	U	39.13	U	U	U	U	U	U	U	U	31.07	34.58	**22.74**	U	33.39	U
SGD100614 5	F	Paus	33.32	35.22	U	31.05	U	U	U	33.33	U	U	U	37.9	27.38	28.04	34.53	**22.35**	37.12	31.3
SGD150414 2	F	Paus	36.28	30.24	U	33.04	U	U	U	32.87	U	U	U	38.04	31.89	35.17	U	**21.71**	U	32.43
COR161213.2	F	Paus	32.58	25.59	U	32.7	U	U	U	36.72	U	U	U	U	30.2	33.62	U	**19.13**	U	31.32
COR041013.1	F	Pnat	U	U	U	U	U	U	U	U	U	U	U	**28.19**	33.07	31.81	U	U	U	U
COR240114.7	F	Pnat	37.54	25.59	U	33.26	U	U	U	34.25	U	U	U	**22.34**	32.68	33.63	U	U	U	U
COR270513.2	F	Pnat	34.49	27.21	U	32.78	U	U	38.74	U	U	38.88	34.78	**19.63**	32.45	33.56	34.84	U	U	U

Extract code	Type	Species	Bbar	Eser	Malc	Mbec	Mbra	Mdau	Mmyo	Mmys	Mnat	Nlei	Nnoc	Pnat	Ppip	Ppyg	Paur	Paus	Rfer	Rhip
COR151113.1	F	Ppip	U	31.05	U	38.2	U	U	U	U	U	U	U	U	**26.31**	U	U	U	U	U
COR151113.5	F	Ppip	U	31.26	U	35.47	U	U	U	U	U	U	U	38.46	**24.26**	34.63	U	U	33.52	32.58
AH210613.4	F	Ppyg	U	31.75	U	38.56	U	U	U	U	36.65	U	U	37.32	32.7	**20.41**	U	U	31.47	34.62
AH 020714.11	F	Ppyg	U	30.99	U	38.1	U	U	U	38.41	U	U	U	U	29.31	**22.19**	35.24	U	U	38.34
AH260914.7	F	Ppyg	U	31.7	U	35.54	U	U	U	U	U	U	U	U	25.83	**20.7**	U	U	U	U
AH260914.8	F	Ppyg	36.12	32.4	U	U	U	U	U	U	U	U	U	35.18	28.71	**18.05**	U	U	U	27.95
AH260914.9	F	Ppyg	U	33.55	U	U	U	U	U	U	U	U	U	U	27.37	**22.06**	U	U	U	U
AH260914.10	F	Ppyg	U	30.8	U	U	U	36.02	U	28.4	U	U	U	34.22	26.35	**19.74**	U	U	U	34.28
COR151113.4	F	Ppyg	U	31.15	U	37.81	U	U	U	U	U	U	U	U	32.5	**25.38**	28.17	U	U	U
COR021113.5	F	Ppyg	39.29	30.77	U	U	U	U	U	U	U	U	33.4	U	30.23	**28.32**	U	U	U	U
COR110613.1	F	Rfer	32.74	27.22	U	31.02	36.26	32.5	38.61	30.07	U	U	U	U	29.12	30.46	27.71	U	**19.03**	29.52
COR121113.2	F	Rfer	34.15	25.58	U	U	U	U	U	38.51	U	U	U	U	33.31	36.07	35.29	U	**19.48**	30.65
COR121113.7	F	Rfer	U	25.44	U	36.48	U	U	U	U	U	U	U	U	32.37	35.11	U	U	**25.85**	31.75
SGD080714-2	F	Rhip	U	U	U	U	U	U	U	33.17	U	U	U	35.3	29.03	28.42	U	U	34.95	**20.75**
SGD140514 5	F	Rhip	U	32.85	U	U	U	U	U	31.93	U	U	U	39.86	31.97	33.16	U	U	U	**19.22**

Appendix 4: Real-time PCR Ct values for bat faecal DNA samples obtained during field surveys of roosts in Ireland. Real-time PCR primers are coded according to target species, as per Appendix 3. Counties are coded as follows: Waterford-WD, Kildare- KE, Galway-GY, Wexford-WX. Species assignment is indicated by Ct value in bold, underlined. DNA extracts are coded as single-pellet or mixed (S/M).

Site	County	No.	Date	Extract code	S/M	Ppip	Ppyg	Pnat	Paur	Mdau	Mnat	Mmys	Mbra	Nlei	Rhip	Rfer
Affane church (RC)	WD	4592	30/08/2012	CB51112.4	S	31.33	31.07	U	U	U	32.47	U	U	U	U	U
	WD	3130	25/06/2013	AH280613.1	S	32.88	**29.56**	U	U	U	U	U	U	U	U	U
	WD	3037	25/06/2013	AH280613.2	S	37	**20.79**	37.08	U	U	U	30.39	U	U	31.93	U
	WD	19617	12/5/2014	AH180614.1	S	28.5	**24.63**	U	U	U	U	U	U	U	34.14	U
Affane Church (C of I)	WD	19620	19/5/2014	AH180614.4	S	29.27	28.15	36.35	U	U	U	33.08	U	U	34	32.77
Aglish Church (RC)	WD	19897	14/08/2014	AH041014.3	M	27.19	29.99	33.98	36.91	35.78	U	37.45	37.32	**23.4**	32.09	31.43
Ardmore Church (C of I)	WD	5092	06/06/2012	COR120612.10	S	31.85	32.61	U	**24.28**	U	U	30.68	U	U	U	U
Ballyduff Lower Church (RC)	WD	18095	30/06/2014	AH 020714.12	M	**21.81**	**23.04**	U	U	U	U	37.05	31.51	U	31.86	U
Ballylaneen Church (RC)	WD	1793	07/09/2012	CB51112.5	S	**26.16**	32.38	U	U	U	U	36.83	U	U	U	U
	WD	19601	23/07/2014	AH181014.10	S	35.83	33.99	U	U	U	U	**25.9**	U	U	U	U
Ballynameelagh Church (RC)	WD	3058	18/07/2013	AH270913.5	S	31.8	**22.22**	37.52	U	U	U	34.15	U	U	31.1	U
Ballysaggart Church (RC)	WD	18096	30/06/2014	AH 020714.13	M	**20.92**	26.11	U	U	34.78	U	31.85	28.08	U	31.72	30.34
Cappoquin Church	WD	19608	30/07/2014	AH181014.14	S	27.26	**22.64**	34.83	U	36.81	U	37.45	31.49	U	29.05	U
Clashmore Church (RC)	WD	1272	27/08/2012	CB231012.11	S	30.09	32.15	U	**23.06**	U	U	33.68	U	U	33.97	U
	WD	1271	27/08/2012	CB231012.10	S	31.41	30.49	U	U	U	U	U	U	U	U	U
	WD	19898	14/08/2014	AH041014.4	M	U	**28.1**	U	U	U	U	U	U	U	U	36.18
Clonea Church (RC)	WD	3032	27/06/2013	AH280613.6	S	31.63	**26.94**	38.4	U	U	U	U	U	U	U	U
	WD	18081	12/6/2014	AH180614.12	S	27.79	26.9	38.21	37.78	38.12	U	U	U	U	37.17	32.45
	WD	18082	12/6/2014	AH180614.13	S	U	**23.76**	34.62	U	32.04	U	30.58	U	U	31.44	30.84
Colligan Church (RC)	WD	5047	27/08/2012	CB221012.10	S	31.46	30.44	39.28	U	U	32.03	U	U	U	U	U
	WD	5046	27/08/2012	CB221012.9	S	32.53	**24.98**	U	U	U	U	U	U	U	31.03	U
	WD	5045	27/08/2012	CB221012.8	S	30.62	**27.03**	U	U	U	U	U	U	U	37.26	U
	WD	18087	24/06/2014	AH 020714.4	M	26.59	28.86	U	U	U	U	27.88	U	U	U	U
Comeragh Church (C of I)	WD	3022	23/07/2013	AH270913.7	S	31.61	**26.95**	U	U	U	U	U	U	U	32.72	31.06
	WD	19597	23/07/2014	AH181014.6	S	27.56	**19.98**	34.83	31.37	U	U	U	31.25	U	30.44	32.28
Corbally Church (RC)	WD	5042	03/09/2012	CB2012.5	S	31.9	31.6	U	U	U	U	U	U	U	U	32.61
	WD	19621	19/5/2014	AH180614.5	S	**26.04**	27.31	U	U	33.04	U	U	33.17	U	U	33.08
Dunhill Church (RC)	WD	817	27/08/2012	CB231012.8	S	32.42	**29.2**	U	U	U	U	U	U	U	U	U
	WD	818	27/08/2012	CB231012.9	S	30.45	**25.77**	U	U	U	U	U	U	U	36.57	U
	WD	19599	23/07/2014	AH181014.8	M	U	**23.48**	U	U	U	U	U	U	U	U	32.37
Dunmore East Church (C of I)	WD	3045	20/09/2013	AH301113.4	S	32.3	31.77	U	**23.86**	U	U	36.53	35.8	U	31.18	U
	WD	19622	19/5/2014	AH180614.6	S	28.65	27.74	38.39	32.06	U	U	32.01	34.44	U	33.05	31.18

Site	County	No.	Date	Extract code	S/M	Ppip	Ppyg	Pnat	Paur	Mdau	Mnat	Mmys	Mbra	Nlei	Rhip	Rfer
Faha Church (RC)	WD	1813	07/09/2012	CB51112.6	S	30.28	31.47	U	U	U	U	U	U	U	U	U
Fenor Church (RC)	WD	5089	06/06/2012	COR120612.7	S	U	U	U	**25.04**	U	U	U	U	U	U	U
	WD	815	27/08/2012	CB231012.6	S	29.82	30.15	U	**29.03**	U	U	U	U	U	U	U
	WD	816	27/08/2012	CB231012.7	S	30.87	33.46	U	**21.32**	U	U	U	U	U	33.79	U
	WD	18092	24/06/2014	AH 020714.9	M	31.96	30.92	U	**21.11**	U	U	32.78	U	U	33.06	U
Fountain Church (RC)	WD	3066	25/06/2013	AH280613.4	S	32.49	32.82	U	U	U	U	**25.96**	U	U	33.39	U
Garranbane Church (RC)	WD	5041	07/09/2012	CB2012.4	S	30.51	**26.1**	U	U	U	U	U	U	U	U	U
	WD	19602	23/07/2014	AH181014.11	M	**20.89**	31.71	U	33.44	U	U	U	27.83	U	34.11	U
Glendine Church (RC)	WD	3048	25/06/2013	AH280613.3	S	33.03	U	U	U	U	U	32.86	U	U	U	U
	WD	19899	14/08/2014	AH041014.5	S	31.83	33.18	U	U	U	U	U	U	U	U	U
Grange Church (RC)	WD	1173	27/08/2012	CB51112.8	S	32.63	30.37	U	U	U	U	37.27	U	U	U	U
Kilgobnet Church (RC)	WD	5048	27/08/2012	CB221012.11	S	31.55	31.45	U	U	U	U	U	U	U	U	U
	WD	3021	24/07/2013	AH270913.6	S	**21.13**	32.9	U	38.13	U	U	U	U	U	32.47	33.72
	WD	18088	24/06/2014	AH 020714.5	M	**18.93**	27.45	38.13	30.34	U	U	39.35	28.83	U	U	38.26
Kilmeaden Church (C of I)	WD	19593	07/07/2014	ES090914.20	S	U	**23.86**	U	U	U	U	U	U	U	U	U
Kilronan Church (C of I)	WD	6087	15/03/2012	COR020712.8	S	31.9	33.45	U	**23.92**	U	U	35.02	U	U	U	U
	WD	5043	27/08/2012	CB2012.6	S	32.26	35.71	U	U	U	U	30.43	U	U	U	U
	WD	19569	09/04/2014	AH170514.2	S	31.06	30.61	34.2	34.51	33.2	U	26.36	U	U	U	29.61
	WD	19603	23/07/2014	AH181014.12	M	34.13	**18.28**	28.83	U	38.2	U	32.99	31.19	U	36.58	U
Kilrossanty Church (RC)	WD	19598	23/07/2014	AH181014.7	M	**23.02**	**20.79**	33.72	31.95	37	U	38.39	29.06	U	U	32.55
Kilwatermoy Church (RC)	WD	4591	30/08/2012	CB51112.3	S	29.17	**23.4**	38.5	U	U	U	U	U	U	U	U
	WD	19610	30/07/2014	AH181014.16	M	U	**23.24**	U	U	U	33.24	U	U	U	U	U
Knockmahon Church (C of I)	WD	814	27/08/2012	CB231012.5	S	30.95	31.4	U	**28.63**	U	U	U	U	U	34.17	U
Lismore Cathedral (C of I)	WD	19606	05/08/2014	AH041014.1	M	31.85	32.03	U	U	U	U	**26.04**	U	U	32.83	U
Modeligo Church (RC)	WD	19607	30/07/2014	AH181014.13	S	32.94	29.16	U	U	U	U	**21.52**	30.93	U	31.13	U
Mountstewart Church (RC)	WD	3131	25/06/2013	AH280613.5	S	U	**27.61**	U	U	U	U	U	U	U	U	U
	WD	18099	30/06/2014	AH 020714.16	M	35.66	**23.73**	U	U	U	U	U	U	U	U	U
Nire Church (RC)	WD	5082	15/03/2012	COR020712.7	S	32.22	33.62	U	U	U	U	U	U	U	U	33.4
	WD	19600	23/07/2014	AH181014.9	M	**21.28**	U	U	**23.18**	**17.64**	37.02	U	28.35	U	U	U
Old Parish Church (RC)	WD	19900	14/08/2014	AH041014.6	S	**23.11**	38.75	U	U	U	U	U	33.76	U	U	U
Piltown Church (C of I)	WD	5091	06/06/2012	AH021214.1	S	31.82	34.86	U	**27.79**	U	U	U	U	U	U	35.4
Portlaw Church (C of I)	WD	5090	06/06/2012	AH021214.2	S	37.14	U	U	**28.71**	U	U	U	U	U	U	34.21
Portlaw Church (RC)	WD	5039	03/09/2012	AH021214.3	S	32.52	31.76	U	U	U	U	U	U	U	34.27	U
	WD	18084	22/06/2014	AH021214.4	M	33.48	31.22	38.44	**20.78**	U	U	U	U	U	U	33.58
Rossmire Church (C of I)	WD	5038	03/09/2012	AH021214.5	S	29.15	**23.03**	U	U	U	U	35.41	36.49	U	U	34.23
Stradbally Church (C of I)	WD	18090	24/06/2014	COR120612.9	S	**20.19**	28.12	37.12	U	38.7	U	U	U	U	31.73	U

Site	County	No.	Date	Extract code	S/M	Ppip	Ppyg	Pnat	Paur	Mdau	Mnat	Mmys	Mbra	Nlei	Rhip	Rfer
Stradbally Church (RC)	WD	18091	24/06/2014	COR120612.8	S	**26.53**	34.54	U	U	U	U	31.43	U	U	U	U
Tallow Church (RC)	WD	19609	30/07/2014	CB2012.2	M	**22.62**	**26.01**	U	U	U	U	U	U	U	U	U
Villierstown Church (C of I)	WD	18098	30/06/2014	AH 020714.1	M	**23.58**	U	U	U	U	U	U	33.89	U	U	U
Windgap Church (RC)	WD	3034	27/06/2013	CB221012.1	S	33.16	35.1	U	U	U	U	33.27	U	U	33.31	U
Lismore bat box 2	WD	18086	19/5/2014	AH180614.2	S	28.17	**17.54**	29.32	U	33.38	U	38.83	U	U	U	U
Lismore bat box 3	WD	18093	19/5/2014	AH180614.3	S	28.53	**19.28**	32.43	U	34.4	U	U	U	U	U	U
Lismore bat box 5	WD	19618	24/06/2014	AH 020714.3	M	29.05	**21.84**	35.33	37.27	U	U	32.08	U	U	U	U
Lismore bat box 6	WD	19619	28/06/2014	AH 020714.10	M	28.82	**18.74**	U	U	U	U	U	U	U	U	U
Clonegam Lodge	WD	5040	03/09/2012	CB2012.3	S	30.03	32.05	U	U	U	U	**24.55**	U	U	U	33.5
	WD	18076	12/6/2014	AH180614.7	S	U	30.73	U	U	U	U	34.31	U	U	U	U
	WD	18077	12/6/2014	AH180614.8	S	39.94	32.21	U	**29.51**	U	U	U	U	U	U	33.19
	WD	18079	12/6/2014	AH180614.10	S	33.19	30.52	U	U	U	U	**17.63**	U	U	U	38
	WD	18080	12/6/2014	AH180614.11	S	28.1	28.67	U	38.44	U	U	U	U	U	31.96	32.96
	WD	19613	08/07/2014	AH181014.2	M	35.05	**24.83**	U	U	U	U	U	30.97	U	U	U
Old depot, Toor Wood	WD	3031	14/02/2013	ES060313.7	S	33.95	33.08	U	**25.83**	U	U	31.51	U	U	U	U
	WD	3041	18/07/2013	AH270913.3	S	33.76	31.78	U	**23.83**	U	U	38.75	U	U	33.67	U
	WD	3046	18/07/2013	AH270913.4	S	34.37	35.09	U	U	U	U	33.89	U	U	34.24	34.27
	WD	19570	09/04/2013	AH170514.3	S	28.18	28.02	38.07	**20.29**	U	U	U	U	U	32.37	U
	WD	19612	08/07/2014	AH181014.1	M	U	U	U	**21.21**	U	37.32	**20.74**	U	U	31.96	U
	WD	19594	08/07/2014	AH181014.3	M	30.2	30.95	U	**21.18**	U	**20.54**	**18.79**	U	U	U	U
	WD	14020	18/09/2014	AH181014.18	M	30.44	35.57	U	**21.73**	U	U	33.38	U	U	U	U
Summerville gate lodge	WD	3071	28/06/2013	AH270913.1	S	**29.24**	33.83	U	U	U	U	U	U	U	U	U
Summerville House	WD	3044	28/06/2013	AH270913.2	S	32.34	**23.33**	U	U	U	U	U	U	U	33.42	U
Grallagh Bridge, River Licky	WD	3042	24/07/2013	AH270913.8	S	35.19	35.86	U	U	U	**20.4**	32.49	U	U	31.78	30.91
	WD	19611	30/07/2014	AH181014.17	S	U	30.68	U	U	U	21.84	**19.92**	U	U	U	U
Private house, Aglish	WD	660	12/11/2013	AH301113.7	S	33.96	**18.69**	U	U	U	U	U	U	U	U	32.84
Outbuilding (front), Aglish	WD	656	12/11/2013	AH301113.5	S	30.13	**19.21**	U	U	U	U	U	U	U	33.35	U
Outbuilding (back), Aglish	WD	659	12/11/2013	AH301113.6	S	U	32.13	U	U	U	U	U	U	U	36.88	U
Belle Lake National School	WD	18085	22/06/2014	AH 020714.2	M	29.03	**22.59**	35.48	U	U	U	U	U	U	U	U
Dromana Gate	WD	18097	30/06/2014	AH 020714.14	M	24.65	**19.72**	U	U	32.85	U	31.74	U	U	31.83	32.2
Ballysaggart Towers Gate	WD	19592	05/07/2014	ES090914.19	S	U	U	U	33.49	U	U	U	U	U	U	U
Farm shed, Woodstown	WD	19616	07/08/2014	AH041014.2	M	29.17	31.97	37.77	**18.4**	U	U	U	U	U	33.26	U
Lismore Cave	WD	3063	11/02/2013	AH150313.9	S	31.5	36.93	U	U	U	U	U	U	U	29.79	33.53
	WD	19277a	04/02/2014	AH150214.1	S	30.5	35.6	U	U	U	**29.37**	U	U	U	U	U
	WD	19277b	04/02/2014	AH150214.2	S	29.42	37.18	U	U	U	**26.61**	34.08	U	U	U	U
	WD	19596	14/7/2014	AH181014.4	M	27.72	32.4	37.85	**22.62**	U	**23.08**	28.59	U	U	35.38	U

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Carrigmurrish Cave	WD	3067	11/02/2013	AH150313.1	M	30.36	32.78	36.24	U	U	U	32.31	U	U	31.34	U
	WD	3057	11/02/2013	AH150313.2	M	32.7	32.8	U	U	U	U	U	U	U	U	U
	WD	19283	11/03/2014	AH050414.3	S	35.18	33.34	U	U	U	U	29.07	U	U	U	U
	WD	19284	11/03/2014	AH050414.4	S	29.44	U	U	U	U	U	28.69	U	U	U	U
	WD	19285	11/03/2014	AH050414.5	S	35.4	U	36.56	U	U	U	28.3	U	U	U	U
Ballynamintra Cave	WD	3070	11/02/2013	AH150313.5	S	31.57	30.93	U	U	U	33.39	33.27	U	U	31.56	31.33
	WD	3054	11/02/2013	AH150313.6	S	29.74	31.79	U	U	U	U	33.18	U	U	U	33.3
	WD	3062	11/02/2013	AH150313.3	S	U	33.03	U	U	U	U	U	U	U	U	33.36
	WD	3055	11/02/2013	AH150313.8	S	34.01	29.97	U	U	U	U	U	U	U	U	U
	WD	3064	11/02/2013	AH150313.4	S	34.22	35.11	U	U	38.23	U	U	U	U	34.51	30.02
	WD	3059	11/02/2013	AH150313.7	S	31.59	34.17	U	35.33	U	U	U	U	U	31.8	31.13
	WD	19281	11/03/2014	AH050414.1	S	U	U	U	U	U	U	28.97	U	U	U	U
	WD	19282	11/03/2014	AH050414.2	S	U	U	U	U	U	U	29.64	U	U	U	U
	WD	19905	23/08/2014	AH260914.5	M	29.65	37.61	U	U	U	**22.63**	U	U	U	31.53	U
Ballynahemery Cave	WD	3039	26/02/2013	AH210613.5	S	U	34.97	U	U	U	**30.42**	U	U	U	U	U
Comeragh sawmill tunnel	WD	3181b	05/03/2013	SM50313.1	S	31.6	38.24	U	U	U	U	U	U	U	38.68	29.8
	WD	3181a	05/03/2013	SM50313.2	S	32.1	37	U	U	U	35.63	U	U	U	33.85	36.79
	WD	19568	09/04/2014	AH170514.1	S	32.89	32.17	38.2	U	U	**28.35**	28.19	U	U	U	31.41
Durrow railway tunnel	WD	18089	24/06/2014	AH 020714.6	M	26.11	27.37	37.31	U	U	33.31	33.68	37.49	U	32.47	U
Kildangan Church (RC)	KE	14023	2014	AH221014.6	S	28.81	**20.35**	31.7	U	U	U	U	U	U	31.17	U
	KE	1176	10/06/2013	AH270913.9	M	36.41	**21.29**	35.89	U	U	U	35.55	U	U	32.67	31.46
St Peter's Church (RC)	KE	1177	2013	AH270913.10	M	33.36	34.05	U	U	35.64	**24.35**	U	U	U	U	30.65
Suncroft Church (RC)	KE	1178	2013	AH270913.11	M	**22.29**	35.42	U	U	U	U	U	U	U	U	U
Kilcok Church (RC)	KE	1179	2013	AH270913.12	M	35.31	31.91	38.75	**16.94**	U	U	38.21	U	U	36.56	U
Fox House Church (RC)	KE	1180	2013	AH270913.13	M	**19.97**	33.07	38.83	**31.6**	U	33.11	33.02	U	U	U	U
Clogharinka Church (RC)	KE	1181	06/06/2013	AH270913.14	M	32.14	32.92	U	**20.46**	U	U	U	U	U	32.7	U
	KE	14025	2014	AH221014.8	M	32.43	32.29	U	**19.79**	U	U	U	U	U	U	U
Kill Church (C of I)	KE	14018	2014	AH221014.2	M	34.59	33.1	U	**20.44**	U	U	35.37	U	U	U	U
Rathmore Church (C of I)	KE	14019	2014	AH221014.3	M	**20.24**	34.43	U	**18.09**	U	U	U	32.69	U	U	U
Donadea bat box 1	KE	18217	2013	AH150214.7	S	**25.24**	33.1	38.86	U	U	U	U	U	U	U	U
	KE	14031	2014	AH221014.14	M	29.41	30.09	34.04	38.31	34.09	U	31.35	U	**24.2**	38.47	31.1
Donadea bat box. 2	KE	14030	2014	AH221014.13	M	31.34	33.04	37.45	36.06	35	U	31.21	36.39	**20.9**	U	U
Donadea bat box 3	KE	14029	2014	AH221014.12	M	32.1	32.16	35.43	U	U	U	U	U	**23.8**	37.07	U
Donadea bat box 4	KE	19278	2012	AH150214.3	S	31.08	31.29	U	U	U	U	U	U	U	U	U
	KE	18218	2013	AH150214.8	S	**26.08**	33.79	U	U	U	U	U	U	U	U	U
Donadea bat box 5	KE	14028	2014	AH221014.11	M	32.06	32.39	37.4	U	U	U	U	U	**26.3**	31.71	37.06

Site	County	No.	Date	Extract code	S/M	Ppip	Ppyg	Pnat	Paur	Mdau	Mnat	Mmys	Mbra	Nlei	Rhip	Rfer
Donadea bat box 6	KE	18219	2013	AH150214.9	M	**25.64**	31.39	U	U	U	U	32.02	U	**26.9**	U	U
	KE	14047	2014	AH251114.2	M	32.46	30	U	U	U	U	30.31	U	**27.6**	34.25	33.68
Donadea bat box 7	KE	14027	2014	AH221014.10	M	31.38	31.17	U	U	U	U	36.05	U	**20.3**	U	U
Donadea bat box 8	KE	19279a	2012	AH150214.4	S	29.39	**24.02**	U	U	U	U	33.68	U	U	U	U
	KE	19279b	2012	AH150214.5	S	30.21	**21.54**	U	U	U	U	32.41	U	U	U	U
	KE	14026	2014	AH221014.9	M	U	**27.33**	U	U	U	U	U	U	**23.4**	33.79	U
Donadea bat box 9	KE	18220	2013	AH150214.10	M	**24.59**	31.34	U	U	U	U	U	U	**29.7**	U	U
Donadea bat box 10	KE	19280	2012	AH150214.6	S	27.25	**21.62**	U	U	U	U	U	U	U	U	U
	KE	18221	2013	AH150214.11	M	27.14	27.68	38.17	U	U	U	U	U	**33.3**	U	U
Private house, Maynooth	KE	14022	2014	AH221014.5	M	**19.93**	29.54	38.48	U	U	U	U	28.16	U	34.27	U
Clone Church (C of I)	WX	14635	22/05/2015	AH160615.11	M	U	**23.5**	37.08	U	U	U	U	U	U	U	U
Crossabeg Church (RC)	WX	14636	22/05/2015	AH160615.12	M	33.07	36.5	U	**26.82**	U	U	U	U	U	U	U
Litter More Church (RC)	WX	14640	22/05/2015	AH160615.14	M	U	**23.5**	38.46	U	U	U	U	U	U	U	U
Agricultural Museum, Johnstown Castle	WX	14643	04/06/2015	AH160615.15	M	U	**17.65**	29.59	U	U	U	U	U	U	U	U
Monageer Church (RC)	WX	14644	19/05/2015	AH160615.16	M	**21.4**	31.19	U	U	U	U	35.18	28.99	U	32.1	U
Tagoat Church (RC)	WX	14647	10/06/2015	AH160615.17	S	**19.15**	30.46	U	U	37.33	U	32.96	25.11	U	U	U
Private House, Caim	WX	14648	08/06/2015	AH160615.18	M	**20.16**	34.03	U	37.19	U	U	33.74	36.04	U	U	U
Scout Den, Davidstown	WX	19573	10/06/2014	AH180614.14	S	28.91	**23.61**	U	U	U	U	U	U	U	U	U
Portumna bat box 127	GY	19562	06/05/2014	AH170514.5	M	34.31	**17.69**	U	U	U	U	U	U	U	U	U
Portumna bat box 102	GY	19563	06/05/2014	AH170514.6	M	30.8	35.29	U	U	U	U	U	U	U	U	U
Portumna bat box 16	GY	19565	06/05/2014	AH170514.8	M	28.55	**24.91**	U	**24.85**	U	U	28.4	U	U	U	U
Portumna bat box 124	GY	19567	06/05/2014	AH170514.10	M	28.57	**17.53**	30.23	U	U	U	30.57	37.18	U	35.92	30.08
Private cottage, Moyne	GY	19904	29/05/2014	AH260914.4	M	29.9	29.86	U	**17.15**	U	26.11	U	U	U	U	29.96
Aughnanure Castle	GY	14021	2014	AH221014.4	M	30.75	34.52	38.61	U	**20.77**	30.39	U	U	U	30.72	U

Appendix 5: Additional bat roost records obtained by daytime sightings or bat detector surveys.

Site	Species	Method
Affane Church (RC)	*Pipistrellus pygmaeus*	Bat detector
Affane Church (C of I), ruin	*Pipistrellus sp.*	Daytime sighting
Ardmore Church (C of I)	*Pipistrellus pipistrellus*	Bat detector
Dunhill Church (RC)	*Pipistrellus pygmaeus*	Bat detector
Dunmore East Church (C of I)	*Pipistrellus pygmaeus*	Bat detector
Fenor Church (RC)	*Pipistrellus pipistrellus*	Bat detector
Greyfriars Church, ruin	*Pipistrellus pipistrellus*	Bat detector
Kilmeaden Church (C of I)	*Plecotus auritus*	Bat detector
Portlaw Church (C of I)	*Pipistrellus pipistrellus*	Bat detector
Durrow railway tunnel	*Myotis nattereri*	Daytime sighting
Lismore bat box 7	*Pipistrellus pygmaeus*	Daytime sighting
Coolnahorna Bridge	*Myotis daubentonii*	Daytime sighting
Rathlead Bridge	*Myotis sp.*	Daytime sighting
Carriganore House	*Nyctalus leisleri*	Bat detector
Private house, Kilmurry	*Nyctalus leisleri*	Bat detector
Old farm building, Corbally More	*Pipistrellus pipistrellus*	Bat detector
Old depot, Toor Wood	*Nyctalus leisleri*	Daytime sighting

Appendix 6: Microsatellite genotypes of unique *R. hipposideros* individuals identified from each roost surveyed, from south to north. Individuals are coded by roost as follows: DC- Derrycreha, CC- Courtney's Cottage, CU- Curragh Chase, CH- Cullaun House, CO- Corbally, KS- Knockaskibbole, KE- Knockanean, RY- Rylane, TN- Toonagh, DR- Dromore, BA- Ballyallaban, FN- Fiddaun, GD- Garryland, BL- Ballylee, CB- Cloghballymore, MC- Menlo Castle, RH- Ross House, BK- Ballykine, LM- Lough Mask House, IM- Inishmaine, BD- Bunnadober.

	RHC108b	**RHD119**	**RHD102**	**RHD103b**	**RHD113**	**RHD111**	**RHD2**
DC001	232 / 232	151 / 151	251 / 255	184 / 188	211 / 215	286 / 286	142 / 150
DC002	232 / 232	151 / 151	251 / 259	176 / 180	207 / 215	278 / 286	150 / 150
DC003	232 / 232	147 / 151	255 / 263	188 / 188	211 / 219	282 / 286	138 / 142
DC004	232 / 232	147 / 151	251 / 251	176 / 180	211 / 215	282 / 282	146 / 150
DC005	232 / 232	151 / 151	251 / 255	180 / 184	211 / 219	294 / 294	150 / 150
DC006	232 / 232	151 / 151	255 / 267	180 / 180	211 / 211	282 / 294	142 / 154
DC007	232 / 232	151 / 151	251 / 255	188 / 196	207 / 211	278 / 278	150 / 150
DC008	232 / 232	147 / 151	251 / 251	180 / 188	215 / 219	278 / 286	150 / 150
DC009	232 / 232	151 / 151	251 / 259	188 / 188	215 / 223	282 / 290	150 / 150
DC010	232 / 232	151 / 151	251 / 255	180 / 180	211 / 219	282 / 286	150 / 150
DC011	232 / 232	151 / 159	251 / 255	188 / 188	211 / 211	286 / 286	138 / 142
DC012	232 / 232	151 / 151	251 / 251	180 / 188	215 / 215	278 / 282	150 / 150
DC013	232 / 232	151 / 151	251 / 251	176 / 180	207 / 219	278 / 294	138 / 162
DC014	232 / 232	151 / 151	251 / 259	180 / 188	211 / 215	294 / 294	150 / 154
DC015	232 / 232	151 / 151	255 / 255	180 / 180	211 / 219	282 / 286	150 / 150
DC016	232 / 232	151 / 151	251 / 263	180 / 196	207 / 231	278 / 294	138 / 150
DC017	232 / 232	151 / 155	263 / 267	176 / 180	211 / 215	278 / 294	150 / 154
DC018	232 / 236	151 / 151	255 / 259	180 / 192	207 / 215	282 / 286	138 / 150
DC019	232 / 232	151 / 151	255 / 267	180 / 196	219 / 223	278 / 290	138 / 150
DC020	232 / 232	151 / 151	251 / 251	184 / 188	207 / 211	278 / 282	150 / 154
DC021	232 / 232	151 / 151	251 / 251	184 / 188	211 / 211	286 / 294	142 / 150
DC022	232 / 232	151 / 151	251 / 255	176 / 180	223 / 223	278 / 278	150 / 150
DC023	232 / 236	151 / 151	259 / 259	184 / 192	207 / 215	278 / 282	138 / 150
DC024	232 / 232	147 / 151	255 / 263	180 / 188	207 / 211	286 / 294	150 / 150
DC025	232 / 236	151 / 151	251 / 259	180 / 180	211 / 223	286 / 298	138 / 150
DC026	232 / 232	151 / 151	251 / 251	176 / 188	211 / 231	282 / 282	150 / 150
DC027	232 / 232	151 / 151	251 / 255	180 / 196	215 / 223	278 / 294	150 / 154
DC028	232 / 232	151 / 151	251 / 251	184 / 188	211 / 219	282 / 290	138 / 150
DC029	232 / 232	151 / 151	255 / 259	188 / 188	207 / 215	282 / 282	142 / 150
DC030	232 / 236	147 / 151	251 / 255	184 / 188	207 / 211	278 / 286	142 / 142
DC031	232 / 232	151 / 151	255 / 255	180 / 192	211 / 215	278 / 282	150 / 150
DC032	232 / 232	151 / 151	251 / 255	188 / 188	211 / 219	278 / 294	138 / 154
DC033	232 / 232	147 / 155	251 / 259	180 / 192	215 / 219	282 / 290	138 / 146
DC034	232 / 232	151 / 151	251 / 263	196 / 200	219 / 231	278 / 282	150 / 162
DC035	232 / 236	151 / 151	251 / 255	184 / 184	207 / 215	278 / 286	142 / 150
DC036	232 / 232	147 / 151	251 / 267	188 / 188	211 / 219	278 / 278	150 / 150
DC037	232 / 236	151 / 151	251 / 263	188 / 192	207 / 223	282 / 286	138 / 150

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
DC038	232 / 232	151 / 151	255 / 263	180 / 180	211 / 223	278 / 278	150 / 158
DC039	232 / 232	151 / 151	255 / 255	180 / 188	211 / 215	278 / 286	150 / 150
DC040	232 / 236	151 / 151	251 / 251	184 / 184	207 / 223	282 / 294	138 / 146
DC041	232 / 232	151 / 151	251 / 259	180 / 196	211 / 215	282 / 294	138 / 150
DC042	232 / 236	151 / 151	255 / 255	184 / 192	211 / 215	278 / 282	138 / 150
DC043	232 / 232	151 / 151	255 / 259	188 / 188	207 / 211	278 / 286	150 / 150
DC044	232 / 236	151 / 151	251 / 255	188 / 192	207 / 215	278 / 282	142 / 150
DC045	232 / 236	151 / 151	255 / 259	180 / 184	219 / 227	282 / 286	138 / 150
DC046	232 / 236	147 / 151	251 / 259	180 / 188	211 / 219	286 / 286	138 / 142
DC047	232 / 236	151 / 151	259 / 267	188 / 192	207 / 207	282 / 282	150 / 150
DC048	232 / 232	151 / 151	251 / 263	184 / 192	211 / 223	282 / 286	138 / 150
DC049	232 / 232	147 / 151	255 / 255	188 / 188	211 / 223	282 / 290	138 / 142
DC050	232 / 232	151 / 151	263 / 263	180 / 184	211 / 223	282 / 298	138 / 150
DC051	232 / 232	151 / 151	251 / 251	180 / 184	223 / 227	286 / 290	150 / 150
DC052	232 / 232	151 / 151	259 / 267	188 / 196	215 / 219	282 / 294	138 / 162
DC053	232 / 232	147 / 151	255 / 259	176 / 196	215 / 223	286 / 294	142 / 150
DC054	232 / 232	151 / 151	251 / 267	188 / 192	211 / 211	278 / 294	138 / 150
DC055	232 / 232	151 / 151	251 / 263	180 / 188	219 / 223	282 / 286	150 / 158
DC056	232 / 236	147 / 151	255 / 259	180 / 184	207 / 219	282 / 294	138 / 154
DC057	232 / 232	151 / 151	255 / 263	180 / 188	219 / 223	294 / 294	150 / 150
DC058	232 / 232	151 / 155	251 / 263	184 / 196	215 / 215	278 / 294	150 / 150
DC059	232 / 232	151 / 151	251 / 263	184 / 188	215 / 215	278 / 294	138 / 150
DC060	232 / 232	147 / 151	251 / 263	176 / 188	211 / 223	286 / 290	138 / 150
DC061	232 / 232	151 / 151	259 / 263	192 / 196	219 / 223	282 / 282	138 / 150
DC062	232 / 232	151 / 155	251 / 263	188 / 196	215 / 219	278 / 286	150 / 150
DC063	232 / 232	151 / 155	251 / 255	180 / 180	211 / 211	282 / 282	142 / 150
DC064	232 / 232	151 / 151	251 / 259	188 / 188	211 / 215	282 / 282	150 / 150
CC001	232 / 232	151 / 155	255 / 259	176 / 184	211 / 211	282 / 282	138 / 138
CC002	232 / 232	151 / 155	251 / 263	184 / 196	211 / 219	278 / 294	150 / 150
CC003	232 / 232	147 / 151	251 / 255	180 / 188	219 / 223	278 / 286	150 / 158
CC004	232 / 236	159 / 159	255 / 259	180 / 188	211 / 215	282 / 286	138 / 142
CC005	232 / 232	151 / 151	251 / 255	180 / 188	207 / 215	278 / 294	138 / 150
CC006	232 / 232	155 / 155	251 / 255	180 / 196	211 / 227	278 / 286	142 / 150
CC007	232 / 232	147 / 151	259 / 263	176 / 180	211 / 219	278 / 282	150 / 150
CC008	232 / 232	151 / 159	255 / 259	176 / 188	219 / 231	278 / 278	150 / 150
CC009	232 / 232	151 / 151	251 / 259	184 / 188	211 / 223	278 / 290	150 / 150
CC010	232 / 232	147 / 151	255 / 255	188 / 192	219 / 219	278 / 294	150 / 150
CC011	232 / 232	151 / 155	251 / 251	188 / 196	207 / 207	278 / 282	138 / 158
CC012	232 / 236	151 / 151	255 / 259	180 / 184	211 / 215	278 / 298	150 / 154
CC013	232 / 232	151 / 151	251 / 255	180 / 180	219 / 223	278 / 282	150 / 150
CC014	232 / 236	151 / 151	251 / 255	180 / 188	207 / 211	282 / 286	138 / 150
CC015	232 / 232	151 / 151	247 / 251	176 / 188	211 / 215	278 / 286	150 / 158
CU001	236 / 236	151 / 155	251 / 255	188 / 196	211 / 211	290 / 290	142 / 162
CU002	236 / 236	151 / 151	251 / 251	188 / 188	211 / 211	282 / 282	126 / 142
CU003	232 / 232	151 / 151	251 / 251	184 / 188	215 / 219	286 / 286	142 / 146
CU004	232 / 232	151 / 151	251 / 259	188 / 188	211 / 211	282 / 282	126 / 146
CU005	232 / 232	151 / 155	251 / 251	184 / 200	211 / 211	282 / 286	142 / 146

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
CU006	232 / 232	151 / 155	255 / 255	184 / 188	211 / 211	282 / 286	142 / 162
CU007	232 / 236	151 / 155	251 / 251	188 / 196	211 / 215	282 / 282	126 / 142
CU008	232 / 232	147 / 155	251 / 251	184 / 188	211 / 215	282 / 282	126 / 146
CU009	232 / 236	151 / 155	251 / 251	188 / 188	211 / 215	278 / 282	142 / 146
CU010	232 / 232	151 / 155	247 / 255	184 / 196	211 / 211	282 / 290	126 / 142
CU011	232 / 232	151 / 159	251 / 251	188 / 188	211 / 211	278 / 294	150 / 162
CU012	232 / 236	151 / 151	247 / 251	188 / 188	211 / 215	282 / 282	126 / 142
CU013	232 / 232	147 / 151	251 / 255	180 / 184	211 / 215	282 / 294	126 / 162
CU014	232 / 232	151 / 155	247 / 251	188 / 188	215 / 215	282 / 282	142 / 166
CU015	232 / 232	151 / 155	251 / 251	188 / 188	211 / 215	278 / 282	126 / 126
CH001	232 / 232	151 / 151	255 / 255	188 / 196	211 / 223	278 / 286	126 / 138
CH002	232 / 236	143 / 159	255 / 255	188 / 196	215 / 215	282 / 282	134 / 138
CH003	236 / 236	151 / 155	255 / 255	188 / 188	211 / 211	282 / 290	142 / 142
CH004	232 / 232	143 / 151	247 / 251	188 / 196	211 / 215	282 / 290	126 / 126
CH005	232 / 236	143 / 151	251 / 255	188 / 204	223 / 223	286 / 286	130 / 154
CH006	232 / 236	147 / 151	251 / 255	188 / 204	211 / 223	282 / 286	126 / 138
CH007	232 / 236	147 / 151	251 / 251	196 / 196	211 / 215	282 / 286	134 / 142
CH008	232 / 232	147 / 155	251 / 259	180 / 188	215 / 219	278 / 282	126 / 126
CH009	232 / 232	151 / 159	251 / 255	184 / 188	219 / 219	282 / 286	126 / 126
CO001	232 / 232	151 / 151	251 / 251	184 / 188	219 / 223	282 / 282	130 / 134
CO002	232 / 236	147 / 151	247 / 251	184 / 204	215 / 223	286 / 286	138 / 150
CO003	232 / 236	151 / 151	251 / 251	192 / 204	211 / 215	278 / 290	142 / 150
CO004	232 / 236	151 / 151	251 / 255	192 / 192	211 / 215	286 / 290	126 / 150
CO005	232 / 232	147 / 151	251 / 255	184 / 196	211 / 219	282 / 286	126 / 146
CO006	232 / 236	143 / 151	255 / 255	184 / 204	215 / 223	282 / 294	126 / 126
CO007	232 / 232	151 / 159	251 / 255	192 / 204	211 / 215	286 / 286	126 / 126
CO008	232 / 232	151 / 151	255 / 255	188 / 204	215 / 219	286 / 294	126 / 142
CO009	232 / 236	143 / 147	255 / 255	184 / 196	219 / 223	282 / 282	126 / 138
KS001	232 / 232	151 / 151	251 / 251	184 / 188	215 / 219	282 / 286	126 / 142
KS002	232 / 232	151 / 151	251 / 251	180 / 188	211 / 215	282 / 282	150 / 150
KS003	232 / 232	151 / 151	251 / 251	184 / 192	215 / 219	282 / 286	142 / 142
KS004	232 / 232	151 / 151	251 / 251	184 / 188	219 / 219	282 / 282	142 / 150
KE001	232 / 232	151 / 151	247 / 255	188 / 192	219 / 219	286 / 286	138 / 142
KE002	232 / 232	151 / 155	251 / 255	188 / 200	207 / 211	278 / 282	126 / 138
KE003	232 / 232	147 / 151	251 / 255	184 / 204	215 / 215	278 / 294	126 / 126
KE004	232 / 236	143 / 151	247 / 251	188 / 196	211 / 223	286 / 298	126 / 130
KE005	232 / 232	147 / 151	251 / 251	184 / 188	215 / 227	282 / 290	150 / 162
KE006	232 / 236	151 / 159	255 / 255	188 / 192	219 / 223	282 / 290	142 / 142
KE007	232 / 232	147 / 151	251 / 255	188 / 196	211 / 215	282 / 282	142 / 154
RY001	232 / 232	151 / 155	251 / 255	176 / 196	219 / 223	282 / 282	146 / 162
RY002	232 / 236	151 / 151	251 / 259	184 / 196	219 / 227	278 / 282	126 / 126
RY003	232 / 236	151 / 155	251 / 263	188 / 196	215 / 219	290 / 290	126 / 126
RY004	232 / 232	151 / 151	251 / 251	184 / 196	211 / 215	278 / 278	138 / 138
RY005	232 / 232	151 / 151	251 / 255	180 / 188	215 / 215	286 / 286	126 / 154
RY006	232 / 236	151 / 151	247 / 255	196 / 200	215 / 219	286 / 290	126 / 150

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
RY007	232 / 236	147 / 151	251 / 255	184 / 188	211 / 211	282 / 298	126 / 142
RY008	232 / 232	147 / 151	251 / 251	192 / 196	211 / 223	282 / 282	126 / 142
RY009	232 / 232	151 / 151	247 / 259	188 / 188	211 / 219	282 / 286	134 / 142
RY010	232 / 232	147 / 151	247 / 251	184 / 192	215 / 219	282 / 282	126 / 138
RY011	232 / 232	151 / 151	251 / 255	188 / 196	219 / 223	282 / 286	126 / 130
RY012	232 / 236	151 / 155	251 / 255	184 / 188	211 / 215	278 / 286	126 / 162
RY013	232 / 236	151 / 151	255 / 263	188 / 196	215 / 223	282 / 298	126 / 138
RY014	232 / 236	151 / 151	247 / 255	180 / 196	219 / 223	286 / 286	126 / 154
RY015	232 / 232	151 / 151	251 / 255	184 / 188	211 / 223	286 / 286	126 / 130
RY016	232 / 236	151 / 155	251 / 255	184 / 188	211 / 223	286 / 286	126 / 126
RY017	232 / 232	151 / 151	247 / 251	188 / 196	215 / 215	286 / 286	126 / 130
RY018	232 / 232	147 / 151	251 / 251	184 / 188	211 / 215	282 / 286	126 / 130
RY019	232 / 236	151 / 151	251 / 255	184 / 188	211 / 211	286 / 298	126 / 138
RY020	232 / 232	147 / 155	251 / 255	188 / 196	211 / 211	278 / 290	142 / 142
TN001	232 / 232	143 / 159	255 / 255	184 / 188	219 / 223	282 / 282	138 / 142
TN002	232 / 232	147 / 151	251 / 251	180 / 188	215 / 219	290 / 294	130 / 142
TN003	232 / 232	147 / 155	247 / 251	188 / 204	215 / 227	286 / 286	126 / 126
TN004	232 / 232	151 / 151	243 / 255	188 / 192	215 / 223	282 / 286	126 / 150
TN005	232 / 232	151 / 151	247 / 255	184 / 192	211 / 215	282 / 286	130 / 138
TN006	232 / 232	151 / 151	247 / 255	192 / 204	215 / 219	286 / 290	122 / 142
TN007	232 / 232	151 / 151	247 / 255	196 / 204	215 / 227	278 / 286	126 / 142
TN008	232 / 232	151 / 151	251 / 251	196 / 196	211 / 215	286 / 290	130 / 150
TN009	232 / 232	151 / 151	251 / 255	180 / 184	211 / 211	282 / 286	126 / 126
TN010	232 / 232	151 / 151	251 / 255	184 / 192	211 / 211	282 / 286	126 / 162
TN011	232 / 232	151 / 151	251 / 255	184 / 192	211 / 215	278 / 282	142 / 142
TN012	232 / 232	151 / 151	251 / 255	196 / 204	211 / 219	286 / 290	122 / 126
TN013	232 / 232	151 / 151	255 / 255	184 / 196	211 / 215	278 / 286	126 / 142
TN014	232 / 232	151 / 151	255 / 259	188 / 196	215 / 219	282 / 290	150 / 154
TN015	232 / 232	151 / 155	243 / 259	192 / 196	215 / 219	282 / 282	126 / 130
TN016	232 / 232	151 / 155	247 / 255	196 / 204	219 / 227	286 / 286	126 / 142
TN017	232 / 232	151 / 155	255 / 263	188 / 192	215 / 227	286 / 298	130 / 142
TN018	232 / 232	151 / 159	251 / 255	180 / 196	215 / 219	282 / 286	138 / 142
TN019	232 / 232	151 / 159	251 / 255	184 / 188	215 / 219	282 / 286	142 / 142
TN020	232 / 232	159 / 159	251 / 255	184 / 188	211 / 219	282 / 286	130 / 138
TN021	232 / 236	151 / 151	251 / 251	176 / 192	211 / 211	286 / 294	126 / 130
TN022	232 / 236	151 / 151	251 / 251	184 / 188	215 / 219	286 / 286	130 / 142
TN023	232 / 236	151 / 151	251 / 251	188 / 196	211 / 215	286 / 290	130 / 146
TN024	232 / 236	151 / 151	251 / 255	176 / 184	211 / 223	282 / 286	126 / 126
TN025	232 / 236	151 / 151	251 / 255	184 / 196	215 / 219	278 / 290	130 / 134
TN026	232 / 236	151 / 151	251 / 255	204 / 204	211 / 227	286 / 290	126 / 158
TN027	232 / 236	151 / 151	251 / 259	192 / 196	215 / 215	278 / 290	126 / 130
TN028	232 / 236	151 / 151	255 / 255	192 / 204	211 / 219	282 / 298	126 / 130
TN029	232 / 236	151 / 151	255 / 259	196 / 196	211 / 215	282 / 290	130 / 134
TN030	232 / 236	151 / 155	255 / 255	192 / 196	215 / 219	282 / 282	126 / 130

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
TN031	232 / 236	151 / 163	251 / 251	184 / 196	219 / 219	282 / 290	134 / 146
TN032	236 / 236	151 / 151	251 / 255	188 / 204	211 / 211	290 / 290	130 / 134
TN033	232 / 232	147 / 151	243 / 259	192 / 200	215 / 219	282 / 282	126 / 126
TN034	232 / 232	147 / 151	251 / 251	184 / 204	211 / 215	286 / 294	126 / 166
TN035	232 / 232	147 / 151	251 / 255	176 / 196	211 / 215	282 / 294	126 / 146
TN036	232 / 232	147 / 151	255 / 259	204 / 204	211 / 215	286 / 286	130 / 146
TN037	232 / 232	147 / 155	247 / 251	196 / 204	215 / 219	286 / 294	134 / 166
TN038	232 / 232	151 / 151	247 / 255	196 / 204	219 / 227	286 / 286	126 / 142
TN039	232 / 232	151 / 151	251 / 259	176 / 184	219 / 219	290 / 298	142 / 154
TN040	232 / 232	151 / 151	255 / 255	184 / 196	211 / 215	278 / 282	126 / 142
TN041	232 / 232	151 / 151	255 / 259	188 / 196	219 / 223	278 / 282	130 / 134
TN042	232 / 232	151 / 155	251 / 251	192 / 196	215 / 215	278 / 282	126 / 142
TN043	232 / 232	151 / 155	255 / 259	176 / 188	215 / 215	282 / 286	126 / 146
TN044	232 / 232	151 / 159	251 / 259	180 / 184	211 / 219	286 / 290	146 / 162
TN045	232 / 232	159 / 159	251 / 251	188 / 204	211 / 219	282 / 282	138 / 154
TN046	232 / 236	151 / 151	243 / 247	192 / 192	215 / 219	282 / 286	126 / 138
TN047	232 / 236	151 / 151	251 / 251	184 / 196	211 / 219	282 / 286	138 / 142
TN048	232 / 236	151 / 151	251 / 251	188 / 196	211 / 219	286 / 286	142 / 142
TN049	232 / 236	151 / 151	251 / 255	180 / 196	211 / 215	282 / 290	126 / 126
TN050	232 / 236	151 / 151	251 / 255	184 / 196	211 / 215	278 / 282	126 / 142
TN051	232 / 236	151 / 151	251 / 255	184 / 196	211 / 215	282 / 290	126 / 130
TN052	232 / 236	151 / 159	251 / 255	188 / 196	215 / 219	282 / 290	130 / 134
TN053	232 / 236	151 / 159	251 / 259	184 / 196	211 / 219	278 / 290	126 / 134
DR001	232 / 232	151 / 151	247 / 251	204 / 208	215 / 219	282 / 290	126 / 146
DR002	236 / 236	151 / 159	251 / 251	184 / 188	211 / 211	278 / 286	130 / 130
DR003	232 / 232	151 / 151	251 / 255	196 / 196	215 / 219	282 / 286	138 / 154
DR004	232 / 232	151 / 151	251 / 251	188 / 196	215 / 215	278 / 278	126 / 142
DR005	232 / 236	151 / 159	247 / 251	192 / 196	215 / 215	282 / 282	150 / 150
DR006	232 / 236	151 / 151	251 / 251	188 / 192	219 / 219	286 / 286	126 / 142
DR007	232 / 236	151 / 151	251 / 255	188 / 188	215 / 219	278 / 290	142 / 146
DR008	232 / 236	151 / 151	247 / 251	176 / 188	215 / 223	286 / 286	130 / 146
DR009	232 / 232	147 / 151	251 / 255	184 / 200	211 / 215	282 / 282	122 / 122
BA001	232 / 232	151 / 151	251 / 251	180 / 184	215 / 219	274 / 278	126 / 142
BA002	232 / 232	151 / 151	251 / 255	188 / 204	211 / 215	282 / 290	126 / 146
BA003	232 / 232	151 / 151	251 / 255	188 / 204	211 / 219	282 / 294	130 / 130
BA004	232 / 232	151 / 151	247 / 259	192 / 204	215 / 219	278 / 298	150 / 150
BA005	232 / 236	151 / 159	255 / 263	180 / 196	211 / 227	278 / 286	138 / 158
BA006	232 / 232	151 / 155	251 / 255	192 / 204	211 / 219	286 / 294	122 / 130
BA007	232 / 232	147 / 151	251 / 251	196 / 196	215 / 215	286 / 286	126 / 142
BA008	232 / 236	151 / 151	251 / 255	180 / 196	211 / 223	282 / 282	126 / 154
FN001	232 / 236	147 / 147	251 / 251	180 / 188	215 / 215	282 / 282	126 / 146
FN002	232 / 236	151 / 151	255 / 255	180 / 204	219 / 227	278 / 282	130 / 138
FN003	232 / 232	147 / 151	247 / 251	192 / 196	211 / 211	286 / 286	142 / 162
GD001	232 / 240	159 / 163	251 / 251	188 / 192	219 / 223	278 / 282	134 / 142
GD002	232 / 232	151 / 155	255 / 259	188 / 196	211 / 227	286 / 286	134 / 142

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
GD003	232 / 232	151 / 151	251 / 251	176 / 204	215 / 219	278 / 282	126 / 142
GD004	232 / 232	151 / 151	255 / 259	180 / 200	215 / 219	286 / 286	150 / 150
GD005	232 / 232	151 / 151	251 / 255	196 / 196	215 / 223	282 / 286	138 / 138
GD006	232 / 236	147 / 151	251 / 251	184 / 196	215 / 219	290 / 298	138 / 142
GD007	232 / 232	151 / 159	251 / 255	188 / 196	211 / 211	282 / 282	126 / 130
GD008	232 / 232	147 / 151	251 / 251	196 / 196	211 / 219	278 / 282	134 / 138
GD009	232 / 232	151 / 151	255 / 255	184 / 192	211 / 219	282 / 282	130 / 150
GD010	232 / 232	151 / 151	251 / 255	180 / 200	211 / 211	282 / 282	138 / 154
GD011	232 / 232	151 / 151	251 / 251	184 / 204	215 / 215	290 / 290	142 / 154
GD012	232 / 232	151 / 159	251 / 259	184 / 196	211 / 219	282 / 282	150 / 154
GD013	232 / 236	147 / 151	251 / 251	180 / 184	211 / 211	286 / 298	154 / 154
GD014	232 / 236	147 / 151	251 / 259	188 / 200	215 / 223	282 / 286	150 / 162
GD015	232 / 232	147 / 151	251 / 259	180 / 184	211 / 223	282 / 286	138 / 150
GD016	232 / 232	151 / 155	247 / 255	188 / 196	215 / 215	278 / 282	126 / 130
GD017	232 / 232	147 / 151	251 / 251	188 / 192	211 / 215	286 / 290	130 / 142
GD018	232 / 232	147 / 159	251 / 251	184 / 196	211 / 211	282 / 298	138 / 138
GD019	232 / 236	143 / 151	251 / 255	192 / 196	211 / 223	282 / 286	146 / 150
GD020	232 / 236	147 / 147	259 / 263	184 / 196	219 / 219	282 / 302	126 / 130
GD021	232 / 232	147 / 151	251 / 251	180 / 180	211 / 215	282 / 282	126 / 138
BL001	232 / 236	151 / 151	255 / 255	184 / 184	211 / 211	282 / 286	150 / 150
BL002	232 / 236	151 / 151	251 / 251	184 / 196	211 / 211	286 / 286	130 / 162
BL003	232 / 232	151 / 159	255 / 259	176 / 188	211 / 211	282 / 294	126 / 126
BL004	232 / 232	151 / 151	251 / 251	180 / 196	215 / 215	282 / 282	146 / 162
BL005	232 / 232	151 / 151	255 / 255	188 / 192	211 / 223	274 / 286	138 / 146
BL006	232 / 232	151 / 151	251 / 255	188 / 188	211 / 211	282 / 286	130 / 150
BL007	232 / 236	147 / 151	251 / 251	176 / 188	215 / 219	286 / 302	126 / 150
BL008	232 / 232	151 / 151	259 / 259	192 / 196	211 / 211	286 / 294	138 / 162
CB001	232 / 232	151 / 151	251 / 251	184 / 204	215 / 219	290 / 290	126 / 150
CB002	232 / 232	147 / 151	251 / 251	184 / 188	215 / 215	282 / 282	130 / 130
CB003	232 / 236	151 / 151	251 / 259	176 / 196	211 / 211	282 / 298	142 / 142
CB004	232 / 232	151 / 151	251 / 251	184 / 200	207 / 211	290 / 298	138 / 146
CB005	232 / 232	155 / 159	251 / 251	176 / 180	219 / 219	290 / 290	130 / 146
CB006	232 / 232	147 / 151	251 / 251	192 / 196	215 / 215	290 / 302	130 / 138
CB007	232 / 232	147 / 151	251 / 255	180 / 204	215 / 223	282 / 282	126 / 138
CB008	232 / 232	151 / 151	251 / 263	192 / 196	219 / 227	298 / 302	126 / 138
CB009	232 / 232	151 / 151	251 / 251	188 / 192	215 / 215	286 / 290	126 / 142
CB010	232 / 236	151 / 151	251 / 251	184 / 196	215 / 219	286 / 290	126 / 130
CB011	232 / 236	147 / 155	255 / 263	180 / 192	219 / 223	278 / 294	126 / 162
CB012	232 / 232	151 / 151	251 / 251	176 / 188	211 / 215	282 / 282	130 / 150
CB013	232 / 232	151 / 155	243 / 255	184 / 196	211 / 215	282 / 290	122 / 146
CB014	232 / 232	151 / 155	251 / 251	184 / 196	211 / 219	286 / 298	138 / 146
CB015	232 / 232	151 / 155	251 / 251	184 / 188	215 / 223	282 / 286	130 / 150
MC001	232 / 232	155 / 155	255 / 255	188 / 192	215 / 219	294 / 298	162 / 166
MC002	232 / 232	155 / 155	251 / 251	188 / 188	215 / 215	294 / 294	158 / 158
RH001	232 / 232	151 / 155	251 / 255	188 / 192	219 / 227	282 / 286	134 / 134

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
RH002	232 / 232	151 / 155	255 / 263	184 / 188	219 / 219	282 / 290	138 / 166
RH003	232 / 232	151 / 151	255 / 259	188 / 188	219 / 219	282 / 282	134 / 138
RH004	232 / 236	151 / 151	255 / 255	192 / 192	223 / 227	278 / 286	134 / 138
RH005	232 / 232	151 / 155	251 / 259	188 / 188	219 / 219	282 / 286	134 / 134
RH006	232 / 232	151 / 155	251 / 255	192 / 192	219 / 223	274 / 282	134 / 166
RH007	232 / 232	151 / 155	255 / 255	180 / 192	223 / 227	274 / 294	134 / 134
RH008	232 / 232	151 / 151	251 / 255	184 / 188	219 / 219	282 / 282	134 / 166
RH009	236 / 236	151 / 155	251 / 255	188 / 192	219 / 219	278 / 286	150 / 150
RH010	232 / 232	151 / 155	251 / 255	184 / 192	219 / 219	282 / 286	134 / 142
RH011	232 / 232	151 / 159	255 / 255	184 / 188	219 / 219	282 / 286	134 / 162
RH012	232 / 232	151 / 151	251 / 255	180 / 188	219 / 219	282 / 286	134 / 150
RH013	232 / 232	155 / 155	255 / 259	188 / 192	219 / 227	274 / 294	134 / 158
RH014	232 / 236	151 / 151	251 / 255	180 / 184	215 / 219	278 / 282	134 / 150
RH015	232 / 232	151 / 151	251 / 255	188 / 192	227 / 227	282 / 282	138 / 138
BK001	232 / 232	151 / 151	251 / 255	188 / 188	215 / 215	286 / 290	134 / 162
BK002	232 / 232	151 / 151	251 / 259	192 / 192	219 / 219	278 / 298	134 / 166
BK003	232 / 232	151 / 155	255 / 263	188 / 192	215 / 223	274 / 286	138 / 162
BK004	232 / 232	151 / 155	251 / 251	180 / 180	219 / 223	278 / 286	134 / 162
BK005	232 / 232	151 / 155	251 / 259	180 / 188	215 / 227	286 / 290	134 / 162
BK006	232 / 232	155 / 155	251 / 255	180 / 188	215 / 219	274 / 282	138 / 154
BK007	232 / 232	151 / 155	251 / 255	192 / 192	215 / 223	274 / 286	138 / 166
BK008	232 / 232	151 / 155	255 / 255	188 / 188	215 / 219	294 / 294	134 / 166
BK009	232 / 232	151 / 155	255 / 255	180 / 188	215 / 223	274 / 290	142 / 162
BK010	232 / 232	151 / 151	251 / 255	188 / 192	215 / 215	286 / 286	162 / 166
BK011	232 / 232	151 / 155	251 / 255	188 / 192	215 / 227	274 / 286	150 / 166
BK012	232 / 232	155 / 155	251 / 255	180 / 192	215 / 219	286 / 290	142 / 162
BK013	232 / 232	151 / 151	255 / 259	180 / 192	211 / 223	286 / 290	142 / 162
BK014	232 / 232	155 / 155	251 / 251	188 / 188	215 / 223	282 / 290	134 / 166
BK015	232 / 232	151 / 151	251 / 263	180 / 184	215 / 219	274 / 278	158 / 162
BK016	232 / 232	155 / 155	251 / 255	180 / 188	211 / 219	278 / 286	134 / 134
BK017	232 / 232	151 / 155	251 / 255	188 / 188	219 / 219	278 / 282	162 / 166
BK018	232 / 232	151 / 155	251 / 255	184 / 188	219 / 219	290 / 294	146 / 162
BK019	232 / 232	151 / 155	251 / 255	188 / 188	219 / 219	282 / 294	162 / 166
BK020	232 / 232	155 / 155	251 / 259	180 / 192	215 / 215	274 / 286	134 / 166
BK021	232 / 232	151 / 151	251 / 259	184 / 188	211 / 227	282 / 282	134 / 142
BK022	232 / 232	151 / 151	251 / 255	188 / 196	219 / 219	274 / 286	134 / 162
BK023	232 / 232	151 / 155	255 / 255	188 / 188	215 / 219	286 / 294	130 / 154
BK024	232 / 232	151 / 155	251 / 251	188 / 188	215 / 219	282 / 294	162 / 166
BK025	232 / 232	155 / 155	255 / 255	188 / 188	215 / 219	286 / 294	166 / 166
BK026	232 / 232	151 / 155	251 / 255	180 / 188	215 / 227	286 / 286	142 / 166
BK027	232 / 232	151 / 155	255 / 255	188 / 188	215 / 219	286 / 294	130 / 146
BK028	232 / 232	151 / 151	255 / 263	180 / 192	211 / 215	274 / 282	146 / 146
BK029	232 / 232	155 / 155	251 / 255	180 / 188	211 / 219	286 / 290	130 / 166
BK030	232 / 232	151 / 155	251 / 251	188 / 188	223 / 227	278 / 286	162 / 162
BK031	232 / 232	151 / 155	255 / 255	188 / 192	211 / 227	278 / 290	162 / 166
BK032	232 / 232	151 / 155	255 / 255	188 / 188	215 / 223	286 / 294	150 / 166
BK033	232 / 232	155 / 155	251 / 251	188 / 192	215 / 219	286 / 290	166 / 166

	RHC108b	RHD119	RHD102	RHD103b	RHD113	RHD111	RHD2
BK034	232 / 232	155 / 155	255 / 259	188 / 188	215 / 219	286 / 294	130 / 166
BK035	232 / 232	151 / 151	255 / 259	188 / 188	215 / 215	286 / 294	162 / 166
BK036	232 / 232	151 / 155	251 / 251	188 / 188	211 / 215	286 / 290	134 / 134
BK037	232 / 232	151 / 151	255 / 255	188 / 188	219 / 223	274 / 274	162 / 166
BK038	232 / 232	151 / 155	251 / 255	180 / 188	219 / 219	286 / 294	134 / 134
BK039	232 / 232	151 / 155	255 / 255	192 / 192	215 / 219	286 / 294	150 / 162
BK040	232 / 232	151 / 151	251 / 259	180 / 192	215 / 219	278 / 290	134 / 134
BK041	232 / 232	151 / 155	251 / 255	188 / 192	211 / 227	278 / 290	162 / 166
BK042	232 / 232	151 / 151	251 / 255	188 / 188	215 / 219	286 / 294	150 / 166
BK043	232 / 232	151 / 151	255 / 263	180 / 188	219 / 227	282 / 286	162 / 166
BK044	232 / 232	151 / 155	251 / 263	180 / 188	215 / 227	282 / 298	134 / 166
BK045	232 / 232	151 / 155	251 / 251	188 / 188	211 / 215	278 / 290	138 / 162
BK046	232 / 232	151 / 155	251 / 251	188 / 188	211 / 215	278 / 290	162 / 162
BK047	232 / 232	151 / 155	255 / 255	188 / 192	219 / 219	278 / 294	134 / 162
BK048	232 / 232	151 / 155	255 / 259	188 / 192	211 / 215	278 / 282	134 / 162
BK049	232 / 232	151 / 151	251 / 255	180 / 188	215 / 219	282 / 290	154 / 166
BK050	232 / 232	155 / 155	255 / 255	180 / 188	215 / 219	286 / 286	134 / 134
BK051	232 / 232	151 / 155	255 / 255	180 / 192	215 / 219	282 / 282	166 / 166
BK052	232 / 232	151 / 155	251 / 255	192 / 192	215 / 219	286 / 286	130 / 142
BK053	232 / 232	151 / 155	255 / 255	192 / 192	219 / 227	278 / 286	142 / 166
BK054	232 / 232	151 / 155	251 / 255	188 / 188	215 / 223	278 / 286	166 / 166
BK055	232 / 232	151 / 155	255 / 263	180 / 184	215 / 223	274 / 282	134 / 134
BK056	232 / 232	151 / 155	251 / 255	180 / 188	211 / 215	286 / 286	166 / 166
BK057	232 / 232	151 / 155	251 / 255	188 / 192	215 / 219	282 / 282	158 / 162
BK058	232 / 232	151 / 151	255 / 255	180 / 192	215 / 219	274 / 286	134 / 138
BK059	232 / 232	151 / 151	255 / 255	188 / 192	223 / 231	274 / 278	138 / 162
BK060	232 / 232	151 / 155	251 / 251	180 / 188	215 / 215	274 / 286	138 / 162
BK061	232 / 232	151 / 151	251 / 255	188 / 188	219 / 227	274 / 278	134 / 158
BK062	232 / 232	155 / 155	255 / 255	188 / 192	215 / 219	274 / 274	142 / 158
BK063	232 / 232	155 / 155	251 / 255	180 / 188	215 / 223	286 / 286	142 / 162
BK064	232 / 232	151 / 155	251 / 255	180 / 188	215 / 219	274 / 290	142 / 150
BK065	232 / 232	151 / 151	255 / 263	188 / 192	215 / 223	290 / 294	130 / 134
LM001	232 / 232	151 / 151	255 / 259	188 / 188	215 / 223	278 / 294	142 / 162
LM002	232 / 232	155 / 155	255 / 259	192 / 192	219 / 219	286 / 286	142 / 162
IM001	232 / 232	151 / 151	255 / 255	180 / 188	219 / 223	278 / 290	142 / 162
IM002	232 / 232	151 / 151	255 / 255	188 / 192	215 / 219	286 / 294	134 / 142
IM003	232 / 232	151 / 155	255 / 255	180 / 188	215 / 215	274 / 282	134 / 154
IM004	232 / 232	151 / 155	255 / 255	180 / 188	215 / 219	274 / 278	138 / 162
IM005	232 / 232	155 / 155	251 / 255	188 / 188	219 / 219	274 / 286	162 / 166
IM006	232 / 232	151 / 155	255 / 255	188 / 192	219 / 223	278 / 286	154 / 154
BD001	232 / 232	155 / 155	251 / 255	180 / 192	223 / 227	286 / 290	158 / 166
BD002	232 / 232	147 / 151	255 / 255	180 / 188	219 / 219	278 / 286	134 / 162
BD003	232 / 232	151 / 151	251 / 255	188 / 192	211 / 223	286 / 290	134 / 142
BD004	232 / 232	155 / 155	255 / 263	188 / 192	219 / 219	294 / 294	142 / 162
BD005	232 / 232	151 / 155	255 / 255	180 / 188	215 / 219	286 / 290	150 / 162
BD006	232 / 232	151 / 151	251 / 255	188 / 188	219 / 219	286 / 290	150 / 162
BD007	232 / 232	147 / 151	255 / 255	188 / 188	215 / 219	278 / 278	134 / 162

	RHC108b	**RHD119**	**RHD102**	**RHD103b**	**RHD113**	**RHD111**	**RHD2**
BD008	232 / 232	155 / 155	251 / 255	188 / 188	215 / 223	286 / 290	150 / 162
BD009	232 / 232	151 / 155	251 / 255	188 / 192	215 / 215	274 / 286	150 / 162
BD010	232 / 232	155 / 155	251 / 255	188 / 188	215 / 223	278 / 290	134 / 162
BD011	232 / 232	151 / 151	255 / 263	188 / 192	219 / 227	274 / 294	134 / 134
BD012	232 / 232	151 / 155	251 / 255	188 / 192	219 / 223	286 / 294	138 / 162
BD013	232 / 232	151 / 155	251 / 255	184 / 188	211 / 215	286 / 286	134 / 134
BD014	232 / 232	151 / 155	251 / 255	180 / 192	215 / 215	274 / 286	166 / 166
BD015	232 / 232	155 / 155	255 / 255	188 / 192	223 / 227	278 / 290	162 / 166
BD016	232 / 232	151 / 155	255 / 259	188 / 192	215 / 219	290 / 294	134 / 166
BD017	232 / 232	151 / 155	255 / 255	188 / 192	219 / 219	278 / 282	150 / 162
BD018	232 / 232	151 / 155	251 / 255	188 / 188	215 / 223	290 / 294	162 / 166