

The spatial and molecular ecology of Twite *Linaria flavirostris*.

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Thesis submitted for the degree of Master of Research

September 2018.

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Acknowledgments

For their patience, support, and their enthusiasm for a little brown finch, I thank my supervisors Kate Durrant and Angus Davison. I'd also like to thank Martin Collinson (University of Aberdeen), Steve Christmas (University of Liverpool) and Matilda Elgood Field for their helpful advice and comments. I am grateful to the RSPB, particularly Nick Wilkinson and David Wood. I am also grateful to the teams at the NHM Tring, Hein Van Grou and Alex Bond; and Gothenburg university, Ulf Molau and Lars Paterson for specimen loans, their time and helpful conversation.

I'd also like to thank Tom Finch, Tom Reeder, Francis Gilbert and Ahmed Saudi for their assistance with the various statistical and graphical methods applied during the course of this study. Additional specimen data came from Robert C. Faucett at the Burke Museum, Seattle, Yaroslav Redkin at the Russian Zoological Museum, Moscow, & Weiland Heim. Finally, the support from my family and friends has been invaluable during the course of this study and for that I am grateful.

Additional support for the Twite monitoring project came from CEMEX, The Derbyshire Ornithological Society, Calderdale Bird Club & Yorkshire Water.

Abstract

Twite *Linaria flavirostris* have a globally disjunct population, with breeding groups in western Europe and from central to east Asia, yet the phylogeographic consequences of this distribution have remained largely uninvestigated. Furthermore, recent population declines in Western Europe now underpin the need to better understand within-species population limits and migratory ecology. This study sought to investigate connectivity and population structure at differing scales within the Twite's global distribution. Data was accessed from ringing schemes across Europe to infer spatio-temporal trends within the Western European breeding group. Mixing between the endemic British subspecies *L. f. pipilans* and the Scandinavian *L. f. flavirostris* is rare and may be perpetuated by differing migratory ecologies. Weak connectivity within the continental group is considered to be a response to instability in available food during the non-breeding season; the findings also suggests a lack of migratory plasticity within the British group. Two regions of the mitochondrial DNA, cyt-b and COI were sequenced in order to explore population structure across the global distribution. A number of informative sites in the mitochondrial DNA identified three narrowly divergent and relatively invariable haplogroups, a single western European, a closely related central Asian group and a divergent Himalayan group. These data are suggestive of recent population expansion within the closely related European and Central Asian groups and, probably related to Pleistocene glacial cycles. Museum specimens were accessed in an attempt to fill gaps in sampling, however sequences retrieved likely contained contaminating DNA. Over all, this study finds isolation within Western Europe and suggests a revision to current IUCN classifications within the region. Future works should seek more comprehensive sampling within the Asian group in order to better understand population limits and connectivity.

Chapter 1

Introduction

The role of genetic data in conservation biology

Understanding the genetic structure and species limits of a population is a prerequisite to effective conservation. However, the definition of a population is convoluted by geographic variation and the gradual evolution of reproductive isolation in allopatry (Tobias et al 2010). It is for this reason that conservation biology now poses what could be some of the most profound and difficult questions ever faced by science (Pimm 2001); The 'Anthropocene' is an age categorised by an unprecedented rate of extinction (Barnosky et al. 2011), which, if left to continue, will disrupt evolutionary processes long into future (Myers and Knoll 2001). The current progression of extinction is considered to be 50 - 500 times that of the background rate, an estimated 3000 - 5000 species per year (Woodruff 2001). Fundamentally, to conserve a species is to attempt to conserve the genetic diversity of an organism, accumulated over the past 3.5 billion years on earth (Eisner et al. 1995) and so, genetic diversity is one of the three forms of biodiversity recognised by the International Union for Conservation of Nature and Natural Resources (IUCN) (Mcneely et al. 1990). However, the IUCN red list of threatened species classification currently lacks an explicit criterion which considers intraspecific genetic diversity, and therefore may not fully account for the ability of a species to adapt to environmental change (Rivers et al. 2014). Environmental change is perhaps one of the biggest threats to biodiversity, and to birds in particular (Crick 2004; Jetz et al. Dobson 2007; Pearce-Higgins and Green 2014; Mainwaring et al. 2017), habitat destruction mainly through urban development or climatic change (Pimm and Raven 2000; Parmesan and Yohe 2003). To reflect this, the IUCN has two measures of range, Extent of occurrence (EOO) and Area of Occupancy (AOO), but the genetic

consequences of range loss and fragmentation are largely neglected (Rivers et al. 2014). The current IUCN red list has classified 11 122 species of bird, of which ~13% (1469) are Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) to extinction. Moreover, according to the IUCN, 20% (2227) of the worlds birds are threatened by habitat destruction and are therefore likely to suffer some degree of fragmentation (IUCN 2017). This is significant as a high proportion of global and local conservation effort is directed by the conservation status of a species, with the most threatened given priority (Possingham et al. 2002). Although this threat-led approach is rarely viable in the long term, as limits to resources prevent equality of treatment and present difficult decisions on which species take priority (Bottrill et al. 2008; Redding and Mooers 2015), it does highlight the need for appropriate classification of species limits within fragmented populations or habitats. Understanding the intraspecific taxonomic affiliations in isolated or fragmented species is an essential step towards their successful conservation (Barrowclough et al. 2016), and therefore the ability to identify genetically isolated groups is fundamental to population scale conservation. The identification of such groups relies not only on the interpretation of current ecology and distribution, but that of evolutionary history.

The biogeography of disjunct distributions

Glaciation events, particularly during the late Pleistocene, have shaped the distribution of species and, in turn, their phylogenetic relationships (Mayr 1942; Mayr 1963). Among the vertebrates, birds may have experienced exceptional divergence following the Pleistocene glaciations (Bermingham et al. 1992). However, the extent to which glaciation shaped speciation in birds is contentious, particularly its role in Nearctic passerine lineages (Klicka and Zink 1997; Lovette 2005). Most authors now believe that the Pleistocene was unlikely to be significant in terms of species formation (Klicka and Zink 1997; Lovette 2005; Finlayson 2009). Instead, dramatic changes to habitat and global biogeography better characterise the period (Finlayson 2009), with genetic divergence coming later as a result of these newly forged distributions. The last ice age was followed by a period of post-glacial re-colonisation of vegetation which in turn,

resulted in a shift to higher latitudes for the Eurasian avifauna as the ice sheets receded to the north (Newton 2013). This was a consequence of rapid glacial recession and ablation during the late Pleistocene, which left a belt of varied topography and heterogeneous habitat from the Iberian Peninsula in the west, across the Eurasian landmass to the Chinese Himalaya in the east (Finlayson 2004). It was this belt of continuous refugia habitat that allowed species to spread between glaciation events across the Palearctic from the expanse of lowland steppe-tundra (boreal belt) to the north. This 'refugium theory' of the formative biogeography of disjunct distributions suggests that species which colonised suitable habitat prior to the late Pleistocene glacial period were then isolated by advancing ice sheets (Fok, Wade, and Parkin 2002; Newton 2003). For example, species which became ubiquitous in the homogeneous northern boreal belt after glacial retreat, were then forced into suitable refugia habitat in the heterogeneous mid-latitudinal belt during glacial expansion, leaving discontinuities in distributions (Newton 2003). The resulting discontinuities in range are known as a population disjunction, and individuals as belonging to a disjunct population. These are defined by discrete intraspecific populations occupying what is apparently a region of continuous suitable habitat (Newton 2003), such as the distributions of the Marsh tit *Parus palustris* (Tritsch et al. 2017), Twite *Linaria flavirostris* (Newton 1972; Cramp and Perrins 1994; Brown and Grice 2005) and the Azure-winged Magpie *Cyanopica cyana* (Fok et al. 2002)..

In the present-day Palearctic, Twite occupy a high/mid-latitudinal range, breeding in boreal regions (60-70°N) of Europe and in the mid-latitude belt (~40°N) of central Asia (Finlayson 2009) and occupy a broad longitudinal range, from around 10°W to 110°E. This distribution is defined by a large gap of absence (~3,000 km), or disjunction between two populations with differing ecologies and habitat requirements (figure 1.1). The Azure-winged magpie may be the only species with a more extreme disjunction between extant populations (Brown and Grice 2005). Although the Twite and the Azure-winged magpie have populations on either side of the Eurasian landmass, the distribution of Twite covers a large area of Asia where the Azure-winged magpie is

absent. Suggestions that the disjunction in the range of the Azure-winged magpie was shaped by anthropogenic factors have been found unlikely (Cooper and Voose 1999; Fok et al. 2002). Instead, the discovery of 40 000 year old avian fossil remains, including Azure-winged magpie in Gibraltar (Cooper and Voose 1999; Newton 2003; Finlayson 2004; Finlayson 2009) and, more recently mitochondrial molecular clock estimations, suggest divergence during the late Pleistocene (Fok et al. 2002).

Despite glacial displacement from the mid latitude belt being the most likely driver of Twite biogeography (Newton 2003; Brown and Grice 2005; Finlayson 2009), Twite are absent from the European Pleistocene fossil record. However, Linnet *Linaria cannabina*, the only other member of the Western European *Linaria*, is represented with fossils dating from the mid (0.781 - 0.126 ma, < five sites) and late (0.126 - 0.0117 ma, 21-101 sites) Pleistocene epoch. Iberian Azure-winged magpie are present from the late Pleistocene (< 5 sites) and the Marsh tit from the mid and late Pleistocene (with < 5 sites reported for each stage) (Appendix 2; Finlayson 2009). The disjunct global distribution of Twite is frequently referenced (Newton 1972; Cramp and Perrins 1994; Atkinson 1998; Brown and Grice 2005; Raine et al. 2006a), but little work has previously been carried out on genetic diversity in disjunct avian populations as a consequence of historic fragmentation, and therefore the impact that it may have on phylogeny or ecology has seldom been addressed.

The phylogeographic consequences of glaciation generally are more well studied, and have included Twite. Marthinsen et al. (2008) suggested that Twite divide into two well-supported monophyletic groups based on analysis of the mitochondrial DNA (hereafter mtDNA) control region and microsatellite allele frequencies, which correspond to two recognised subspecies, an Asian and a European ($n = 8$). Molecular studies, focusing on the mtDNA cytochrome b (cyt b) gene, have shown rapid radiation occurring within the Cardueline group during the Miocene/Pliocene epoch boundary (Arnaiz-Villena et al. 1998; Payevsky 2015). Arnaiz-Villena (1999) investigated both *Carduelis* (then including Goldfinches *Carduelis*, Greenfinches *Chloris*, Siskins *Spinus* and Redpolls *Acanthis*) and *Serinus* in terms of the effect glaciation events may have had on diversity within

these genera. The study found that glaciation during the Pliocene had little effect on the speciation of either clade but may have informed sub-speciation (or increased intraspecific phylogenetic diversity) following a rapid radiation into newly available habitats. This finding is corroborated by later work into the comparative phylogeography of five alpine species, including Twite (Qu et al. 2010). This study suggests that two species (the Twite and the Black redstart *Phoenicurus ochruros*) from the Tibetan avifauna maintained stable populations throughout the Last Glacial Maximum (LGM 0.023-0.018 ma) as both occupied a single alpine edge refugium which may have been largely unaffected by glacial ice cover. This finding does not address the disjunction in population, but may explain sub-specific structure within the Tibetan group, concluding that Pliocene climate fluctuations were unlikely to have shaped genetic diversity in Twite and black redstart (Qu et al. 2010).

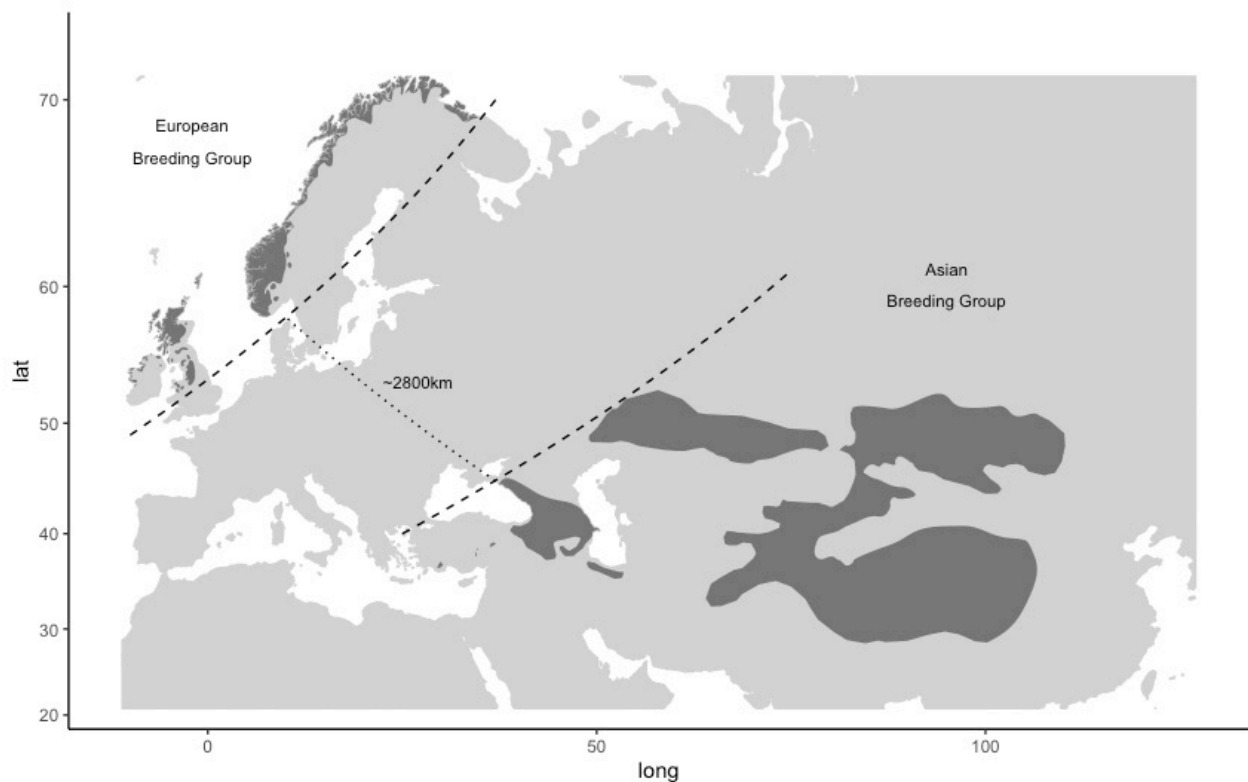


Figure 1.1. The global breeding range of Twite is broken up onto two major centres: a western, European group and an eastern, mid latitudinal Asian group. Note the large gap of absence between the two, ~2800km at its closest point.

Measuring avian phylogeography

Phylogeography is an integrative area of research which combines biogeography, paleoecology and population genetics to resolve how distribution and geographic vicariance effect phylogenetic relationships (Avice 2000; Hickerson et al. 2010). Where Pleistocene glaciation events are held to be formative in species biogeography, those disrupted distributions would later shape the phylogenetic relationships of organisms within a population. For example, during periods of sustained isolation, over many hundreds of generations, novel genetic mutations arise within an isolated group and are precluded from other groups through limited/low dispersal of individuals (Allendorf et al. 2014). Therefore, phylogenetic population structure is expected in populations which, over time, become isolated from one and other preventing mixing of individuals and

subsequent gene flow within the population. In continuous populations, genetic diversity may still exist through historic isolation (and recent secondary contact) or through genetic drift (Allendorf et al. 2014). Phylogenetic structure between species varies from deep phylogenetic breaks (Kryukov et al. 2017) to panmictic (or random mating) populations (Riddle 1996). Likewise, phylogeographic structure can vary between regions; where intraspecific populations at lower latitudes are more likely to exhibit greater evolutionary independence. This increases the likelihood that genetic mutation, recombination or drift will result in a divergence of traits important for reproductive isolation (Martin and McKay 2004). Divergence patterns in closely related taxa (Botero et al. 2014) and subspecies (Weir and Price 2011) are apparently greatest in the temperate zone (Smith et al. 2017). Species living at lower latitudes have a greater phylogeographic structure than those living at higher latitudes, which is related to the age and to the relative stability of environments associated with the formation of diversity at these gradients (Smith et al. 2017).

In order to measure phylogeography a genetic marker is selected and measured between groups within a population and those data compared against current distribution and ecology. MtDNA has been used extensively in the study of evolutionary history and phylogenetic relationships between organisms over the last three decades (Ballard and Whitlock 2004) and has become the most frequently used marker in animal phylogeographic studies (Bermingham et al. 1992; Lerner et al. 2011; Aoki et al. 2018); mtDNA is a small genome present in large quantities within the cell, it is passed down the maternal line and has a higher mutation rate than the nuclear genome (nDNA) (Brown, George, and Wilson 1979; Ballard and Whitlock 2004; Gupta and Bhardwaj 2013; Allio et al. 2017). The use of mtDNA is therefore common in avian phylogenetics. (Kerr et al. 2009; Qu et al. 2010; Johnsen et al. 2010; Zuccon et al. 2012; Aliabadian et al. 2013; Price et al. 2014). Perhaps the most frequent use of mtDNA in phylogeography is the construction of phylogenetic trees from haplotype sequences, which contextualise spatial associations with genetic diversity between taxonomic groups. Generally, the formation of a phylogenetic tree arranges more recent ancestors nested within groups

of taxa with more distant ancestors (Morrison 1996). Much of this research within vertebrates has addressed phylogeographic relationships within the birds (Zuccon et al. 2012; Bermingham et al. 1992; Lerner et al. 2011; Kryukov et al. 2017; Smith et al. 2017; Aoki et al. 2018), which exhibit a high vagility and often breed great distances away from non-breeding territory, thus, recruitment can take place at different points throughout the annual cycle. This can be overcome with the incorporation of demography data such as migratory connectivity metrics which measure the degree of mixing between intraspecific breeding populations during the non-breeding season. Complete isolation (though either distance or niche partitioning) is known as strong connectivity where, association between groups with no barrier to free mixing is known as weak connectivity (Trierweiler et al. 2014; Finch et al. 2015). Although counter-intuitive terminology, connectivity refers to the link between breeding and non-breeding ranges of pairwise individuals and not the social connection between individuals within discrete groups.

The Finch family and the Twite

The true Finches (Aves: Passeriformes: *Fringillidae*) are a diverse family of seed-eaters with a near-cosmopolitan global distribution, absent only from Antarctica and Australia (Sibley and Ahlquist 1990; Cramp and Perrins 1994; Zuccon et al. 2012) and separated from the wider passeroid oscine clade by a vestigial tenth primary remex or, flight feather (Hall and Susanna 2005) and highly specialised bill morphology. The family represents some of the most iconic and familiar generalist bird species which are readily attracted to sub-urban garden feeders such as the Chaffinch *Fringilla coelebs* and Goldfinch *Carduelis Carduelis* (Fuller et al. 2008). Conversely, others are range restricted, highly specialised and often threaten with extinction, such as the the Hawaiian honey-creepers *Drepanidini* (Lerner et al. 2011). Of the 201 currently recognised species, represented by 49 genera, 18% (36) species are at risk of extinction; seven near threatened (NT), 10 vulnerable (VU), nine endangered (EN), seven critically endangered (CR) and three critically endangered, possibly extinct (CR PE), with habitat destruction and fragmentation the primary threat (IUCN 2017).

The Finches divide into two major subfamilies, the monotypic *Fringillinae*, and the *Carduelinae*. The *Carduelinae* are a polyphyletic group of morphologically and behaviourally diverse species (Hall and Moreau 1970; Clement et al 2011; Zuccon et al. 2012; Winkler et al. 2015), representing all but 35 of the 201 currently recognised species within the wider *Fringillidae* family (Winkler et al. 2015). Whilst the *Fringillinae* have remained largely taxonomically stable, in part due to clear physiological and behavioral differences from their sister group (Sushkin 1924; Cramp and Perrins 1994), the complexity of *Cardueline* affiliations have been reflected in their multiple re-interpretations (Fehrer 1995; Nguembock et al. 2009; Zuccon et al. 2012). Most recently, Zuccon *et al.* (2012) recently proposed that the Eurasian *Carduelinae* should be split into several genera, the monophyletic *Linaria* (Twite and Linnets) and the polyphyletic groups of *Acanthis* (the Redpolls), *Spinus* (the Siskins) and *Carduelis* (Goldfinch and Citril finch).

Further changes were proposed to the Serin complex, splitting the group into *Serinus* (the Palearctic Serins and Canaries) and *Crithagra* (the Afrotropical, Serins), a stance which has since been largely adopted by taxonomic authorities (Sangster et al. 2016; Gill and Donsker 2017), and corroborated by Arnaiz-Villena *et al.* (2013). Within the *Carduelinea* the Common (or Mealy)/Lesser Redpoll (*Acanthis flammea/cabaret*) complex is perhaps the best known, and widely discussed in terms of its taxonomy, in part thanks to interest from amateur ornithologists and its association with urban gardens. Despite reported vocal, molecular and behavioural differences (Knox et al. 2001), mtDNA appeared to be unrelated to geographical or traditional taxonomic relationships between proposed Redpoll species, and differences slight when compared with typical sister taxa (Seutin et al. 1995). Marthinsen *et al.* (2008) examined the mtDNA control region and microsatellite markers and found no specific structure in Redpoll mtDNA haplotypes nor allele frequencies compared with the out group (Twite). The Pleistocene glaciation may have also been important in the sub-speciation of the Crossbills *Loxia* (Arnaiz-Villena et al. 2001). mtDNA (cyt b) analysis has revealed the Crossbills are closely related to the Redpolls and therefore deeply nested inside the

Carduelinae, despite morphological distinctions (Arnaiz-Villena et al. 2001; Arnaiz-Villena et al. 2013). Unlike the Redpolls, Crossbills have undergone sympatric speciation in the recent past (Knox 1977). One study on a population of three sympatric breeding species of Crossbill in the Scottish Highlands, The Common Crossbill, *Loxia curvirostris*, The Parrot Crossbill, *L. pytyopsittacus* and the cryptic endemic, Scottish Crossbill, *L. scotica*, found assortative mating, but no mtDNA diversity between the groups (Summers et al. 2007). Examples such as the Redpoll and Crossbill complexes show that sympatric populations of Cardueline finches can maintain genetic structure following secondary contact, but also, in the case of the former, may undergo phenotypic divergence without speciation. Little work has been done on large disjunct populations within the family such as the Twite, likewise gene flow and genetic diversity between the Twite groups is largely unknown.

Conservation status of Twite in Great Britain and Ireland

The Twite is a gregarious Cardueline finch with a disjunct global distribution (Newton 1972; Cramp and Perrins 1994). Twite and the sister taxa, the Common linnet are perhaps one of the most obvious examples of niche partitioning in western Europe (Lack 1974); Twite breed at higher altitudes than the lowland Linnet, associating with mountainous and steppe habitat (BirdLife International 2015). Both species have an exceptionally fine bill, adapted for extracting seeds from grasses and small flowering plants (Newton 1972). Twite rely on a range of food plant species which change across the annual cycle and therefore limit distribution (Raine 2006); in the spring Twite feed almost exclusively on grass seeds such as Pao *Annua* and purple moorgrass *Molinia*. During the breeding season, sorrel species *Rumex*, autumn hawkbit *Leontodon autumnalis* and thistle species *Cirsium* become important (Cramp and Perrins 1994) and their relative abundance dictates subsequent Twite breeding productivity (Raine 2006). Over winter European Twite are partial migrants moving towards coastal and lowland farmland in search of food; here, often in assortative foraging flocks with Shorelark *Eremophila alpestris* and Snow bunting *Plectrophenax nivalis*, they specialise on samphire sp. *Salicornia* and seablite *Suaeda maritima* (Cramp and Perrins 1994; J

Dierschke 2002). The current conservation model for Twite is therefore focused on the restoration of upland meadows with a lesser emphasis on winter food plant abundance, both of which are thought to limit population growth and range (Dierschke 2002; Raine 2006).

With the exception of the endemic Scottish Crossbill *Loxia scotica*, Twite were once the only passerine to breed or winter in Britain in what was considered internationally important numbers (Batten et al. 1990; Brown et al. 1995), and as a result, in Britain and Ireland the endemic race *pipilans* and the continental *flavirostris* have remained on the Red List: Birds of Conservation Concern (furthermore BoCC), since it was established in 1996 (Eaton et al. 2015). This placement on the red list is due to recent population declines and a constriction of the breeding range in England, Wales and Ireland (Brown and Grice 2005; Raine et al. 2009; Eaton et al. 2015). The distribution of breeding Twite in Britain and Ireland has been well documented over several decades (Orford 1973; Sharrock 1976; Gibbons, Reid, and Chapman 1993; Raine et al. 2006a; McLoughlin et al. 2010; Balmer et al. 2013); the 1988-91 Breeding Bird Atlas indicated that Twite are confined to regions of coastal agriculture, notably crofting land in Scotland (Wilkinson and Wilson 2010) as well as the southern half of the English Pennine hills (Gibbons et al. 1993; Raine et al. 2006b), The 2007-2011 Atlas shows little change in spatial occupancy throughout the annual cycle, but a -29% reduction in the number of occupied survey squares within these regions since the 1968-72 survey. These declines are most profound in Ireland, with an -80% reduction in breeding abundance and -19% in Great Britain (Balmer et al. 2013).

The Twite also saw a significant constriction in breeding abundance during the 20th century (Holloway 1989; Langston et al. 2006; Raine et al. 2009). Holloway (1989) suggests that the number of counties in England where Twite were recorded fell by 36% between 1875-1900, further corroborated by Raine (2009) who found that between 1800-2000, Twite had in fact been lost from 8 of 14 (57%) historic breeding counties. When data from the 2007-2011 atlas is added, Twite are now only confirmed breeding in three English counties: Yorkshire, Lancashire and Derbyshire; whilst being

considered possible or probable in a further four: Durham, Northumberland, Cumbria and Greater Manchester respectively, see Table 1.1.

Twite are poorly represented in national monitoring schemes such as the BTO's Breeding Bird Survey (BBS) and Wetland Bird Surveys (WeBS) and thus, numbers breeding and wintering in the Great Britain and Ireland are difficult to quantify (Raine et al. 2009). This is a result of both sampling biases towards areas of more favourable access to volunteer surveyors, low densities and long foraging trips in suitable habitat (Raine et al. 2006b; Wilkinson and Wilson 2010) and a highly fragmented distribution. Dedicated surveys have been carried out and subsequently published, charting the historic decline since Holloway (1996). The first survey was carried out in 1999, and suggested a UK population of 10 000 pairs (95% CI: 6300 – 14 600) with the highest densities in Scotland, which was estimated to hold 94% of the population, leaving 6% split between England, Ireland and Wales (Langston et al. 2006, see table 1.2). Further work was carried out by Raine (2009), but focused on the English breeding group, finding a further range constriction of -83% since the 1999 survey. A revised population survey was carried out in 2013, which estimated the UK Twite population at 7831 pairs (95% CI: 5829 – 10 137), 21% lower but not significant from the 1999 survey, however, Scotland now held 98% of the UK Twite population (7640, 95% CI: 5629 – 9 994) leaving only ~164 pairs in England (95% CI: 76 - 297), a significant decline of 72% since the 1999 survey. The number of Twite pairs in Wales and Northern Ireland were estimated to be at 16 and 18 respectively (Wilkinson et al. 2018). A small survey in 2016 - 2017 (RPB, unpublished) suggests a further decline in both abundance and range of ~52%, and that the English breeding population could be as low as 100 pairs.

Table 1.1. Table showing occurrence of Twite in 15 historic breeding counties. Note that — denotes present, - , rarely encountered but thought to be present and X, population extirpated. a blank cell denotes no records were reported during the time period. NA in the final column denotes that there were no records for the county during the bird atlas survey period, in this case 2007 - 2011. *Prior to its establishment 1974, Greater Manchester was split between counties of Lancashire, Cheshire and the West Riding of Yorkshire and has been treated as such here. Reproduced from (Raine et al. 2009) with an additional column for more recent bird atlas data (Balmer et al. 2013) and a row showing Greater Manchester post its establishment in 1974 (Holland et al. 1984).

County	1800	1900	1910	1920	1930	1940	1950	1960	1970	1980	1990	2000	2007-2011
Northumberland	—	—	—	—	—	—	X			-	X		Probable
Cumbria	—	—	—	—	—	—	—	—	-	-	-	-	Probable
Durham	X								-	-	-	-	Possible
Yorkshire	—	—	—	—	—	—	—	—	—	—	—	—	Confirmed
Lancashire	—	—	—	—	—	—	—	—	—	—	-	-	Confirmed
Cheshire	—	—	—	—	—	—	-X		-	-	-	-	NA
Derbyshire	—	—	—	—	—	—	—	—	—	-	-	-	Confirmed
Nottinghamshire	-	-	X										NA
Staffordshire	—	—	—	-X					-	—	-	X	NA
Warwickshire	X									-	-	X	NA
Worcestershire	—	X											NA
Herefordshire	—	X											NA
Shropshire	—	X											NA
Devon	X	-	-	-	-X								NA
*Greater Manchester									—	—	—	-	Probable

Table 1.2 Results from the 2013 Twite survey by country/region against the 1999 survey data with percentage change. Reproduced from (Wilkinson et al. 2018).

Country/Region	2013 (95% CI)	1999 (95% CI)	Percentage change (95% CI)
Scotland	7,640 (5,629-9,954)	9,361 (5,619-13,793)	-18 (-51, 47)
England	164 (76-297)	587 (325-881)	-72 (-89, -35)
South Pennine Moors (SPA) England.	162 (74-295)	-	-
Northern Ireland	18	10	-
Wales	10 (8-13)	5	-
UK	7831 (5,835-10,143)	9,963 (6,308-14,601)	-21 (-51, 35)

Conservation status within the European union

Outside of the UK and Ireland, Twite have not received the same degree of constant monitoring effort, however some work does exist on their demography and distribution. Within continental Europe Twite breed primarily on the west coast of Norway, associating strongly with sea cliffs. Extending north into the Arctic circle, a lesser number breed in Sweden and Northern Russia. The demography of alpine birds in Europe has been rarely studied despite now facing the acute risks of habitat loss and range contractions associated with current and projected climatic change (Resano-Mayor et al. 2017); this projection may translate to the winter distributions, where the southern limit of the continental Twite group already seems to have receded north over time (Sonvon: Frater 2016; Vergeer and Marx 2018).

Species residing within Europe and the EU27 have been assessed separately by Bird Life international and here Twite are classed as VU (EU27) and LC (Europe and internationally) (BirdLife International 2015). Of 451 species assessed within the EU27, 55 were found to be vulnerable, 16 endangered, 11 critically endangered and one extirpated (regionally extinct), but still residing outside of the region, a further two species are globally extinct.

In terms of the Twite's breeding distribution across eastern Europe, the Asian group marginally extends into eastern Turkey, Georgia, Armenia and Azerbaijan and this in part accounts for the IUCN regional assessment VU classification. Although very little is known about these populations some data has been made available through BirdLife international (2015), see table 1.3.

Table 1.3 Population sizes and dates of census from Europe. Reproduced from supplementary material data supporting the European assessment of Twite for BirdLife international (2015). With an additional column to show the ssp., and inferred group (Western; *pipilans* and *flavirostris*, Eastern/Asian; *brevirostris*/ *korejevi*?).

Territory	ssp.	Size (pairs)	Europe (~%)	Year(s)	Quality	Source
Armenia	brevirostris	15,000 - 25,000	6	2002-2012	medium	ASPB data
Azerbaijan	brevirostris	1,000 - 10,000	1	1996-2000	poor	(BirdLife, 2014)
Finland	flavirostris	0 - 10	<1	2006-2010	medium	(Valkama et al., 2011)
Georgia	brevirostris	breeding	<1	-	-	(Bird life, 2014)
Rep Ireland	pipilans	54 - 110	<1	2005-2008	good	(McLoughlin, 2011)
Norway	flavirostris	100,000 - 500,000	64	2013	poor	(Shimmings et al., 2015)
European Russia	flavirostris	2,000 - 5,000	1	2000-2002	poor	(Belik, 2005)
Sweden	flavirostris	160 - 500	<1	2008-2012	poor	(Ottosson et al., 2012)
Turkey	brevirostris	40,000 - 200,000	26	2013	poor	Cemil Gezgin (Pers comm to Bird life)
United Kingdom	pipilans	6,000 - 15,000	3	1999	medium	

IUCN Conservation status

Internationally Twite are considered Least Concern (LC). The large range size means that Twite do not meet the requirements for Vulnerable (VU) classification, which are: Extent of occurrence $< 20\,000\text{ km}^2$, combined with a declining or fluctuating range size, habitat extent/quality, or population size and small number of locations or severe fragmentation. Currently Twite are estimated to occur over $21\,400\,000\text{ km}^2$ in the breeding season and $8\,410\,000\text{ km}^2$ in the non-breeding season. Although the population is suspected to be decreasing, it does not qualify for vulnerable under the population trend criterion ($> 30\%$ decline over 10 years or three generations). Nor does the population approach the lower threshold for population size ($< 10,000$ mature individuals with a continuing decline estimated to be $> 10\%$ in 10 years or three generations, or with a specified population structure), number of mature individuals was estimated to be within $1\,000\,000 - 19\,999\,999$ in 2012. Despite this evaluation, the quality of the available data is thought to be poor (BirdLife International 2018). The same report lists three habitat types/altitudes for the species (table. 1.4, below), however coastal/estuarine mud flat environs strongly associated with the non-breeding season in Europe are missing.

Table 1.4 Habitats occupied by Twite internationally and their importance according to the IUCN. Reproduced from (BirdLife International 2015).

Habitat (level 1)	Habitat (level 2)	Importance	Occurrence
Grassland	Temperate	Suitable	Breeding
Grassland	Temperate	Suitable	Non-breeding
Rocky areas (eg. inland cliffs, mountain peaks)		Major	Breeding
Rocky areas (eg. inland cliffs, mountain peaks)		Major	Non-breeding
Scrubland	Temperate	Suitable	Breeding
Scrubland	Temperate	Suitable	Non-breeding

As well as habitat the report documents distribution by country (table 1.5, below). Twite occur naturally in 44 countries; 18 Asian and 32 European, and are considered vagrants in another six. Of these, Twite either breed or are resident (meaning here that the whole annual cycle is represented to some extent) in 16 Asian (NB, Russia has been broken into four regions, each counted separately, these are Russia, Central Russia, Asian Russia and European Russia) countries and seven European. Asian census and ringing data is scant, so inferences have been made from European data. The Bird life report states that the European Twite group forms ~10% of the global range, and from this a preliminary estimation is made of the global population size, by assuming similar densities across their range, of 3 280 000 – 15 100 00 mature individuals. However further validation of this estimate is needed (BirdLife International 2015).

Table 1.5 Countries with breeding or regular non-breeding groups of Twite according to the IUCN.

European breeding /resident	Asian breeding/ resident	European Non-breeding	Asian Non-breeding
United Kingdom	Afghanistan	Austria	Iraq
Finland	Armenia	Belarus	Turkmenistan
Lithuania	Azerbaijan	Belgium	
Norway	China	Bulgaria	
European Russia	Georgia	Czech Republic	
Sweden	Iran	Denmark	
	Kazakhstan	Estonia	
	Kyrgyzstan	France	
	Mongolia	Germany	
	Nepal	Hungary	
	Russia	Ireland	
	Asian Russia	Latvia	
	Central Russia	Netherlands	
	Tajikistan	Poland	
	Turkey	Romania	
	Uzbekistan	Switzerland	
		Slovakia	
		Spain	
		Ukraine	

Aims

This study aims to, for the first, time address aspects of Twite ecology and biogeography at varied spatial scales to explore within-species connectivity and population limits. The disjunct population is split between two groups, a European and an Asiatic, however population limits and connectivity between and within these groups are unclear. I first use historic ringing data from the EURING data bank to explore connectivity between the two population centres, alongside local survey data, within the fragmented European range (Chapter 2). In doing so, it is hoped that these results will better inform the implementation of conservation strategies within Europe, as well as filling spatial ecology knowledge gaps for this enigmatic and highly specialised species. I then utilised molecular techniques to further infer connectivity and population structure within the Twite's global range (Chapter 3). I also attempted to fill gaps in available DNA sampling bias with ancient DNA extracted from museum specimens (Chapter 4) and amplified using newly developed primer pairs. These data were then used to build a series haplotype networks in an attempt to quantify relationships between the disjunct breeding groups internationally. I hope that these data in their entirety can be used to inform a more comprehensive IUCN regional assessment of Twite which better reflects localised conservation status.

General Methods

Data collection

Data was requested from the European Union for Bird ringing (Euring), which coordinates bird ringing at a continental scale and maintains the European data bank (furthermore EDB) (DeFeu et al. 2016). The EDB currently holds more than 10 million individual encounter records for 485 species and from schemes in 28 European countries (EURING 2015). Twite data (22 622 records) was accessed first in 2016 and renewed in 2018. This data was used to explore both spatial and temporal relationships between groups, see Spina (1999). Because mark recapture techniques are spatially precise but temporally coarse, often only providing an origin and recovery location with little data on migratory routes (Finch et al. 2017), particularly over sea crossings, data was also accessed from the North Sea Bird Club (hereafter, NSBC). The NSBC was formed in 1979 in collaboration with the university of Aberdeen Ocean laboratory and centre of ecology in order to obtain long-term data from wildlife observed around offshore infrastructure. Although the precise identification of individual data points are impossible to verify, the NSBC hold in excess of 120 000 records of birds, cetaceans and insects reported offshore (NSBC 2018). NSBC Data was accessed to determine the relative occurrence of Twite on offshore infrastructure. Counts for four Anglo/Scandinavian migrants and three partial migrant species were also accessed to contextualise results.

Primary field data was collected in the form of ring recoveries/re-sightings, feather samples and buccal swabs. Twite were captured and ringed with four leg rings, one coded metal ring on the lower left leg, below a plain colour ring as well as a further two plain colour rings on the right leg, or an alpha/numeric coded plastic 'darvic' (see capture and monitoring of live birds); 832 Twite were captured and individually marked between 2013 and June 2018, ~611 in England and ~90 in Scotland, 131 were recaptured individuals. A further 743 were reported based on the individual combination of colour rings. Additional Twite ringing data was accessed from collaborators from the

Twite network working group. Secondary feather samples were provided by collaborators in Scotland, Wales and Sweden (see Field sites; figure 1.2).

Historic toe pad samples were provided by the Natural History Museum (NHM), Tring. Twite specimens were selected based on subspecies and geographic origin (see Museum samples). Additional data was accessed from Gene bank, the Burke Museum, Seattle and Zoological Museum, Moscow university.

Primary field sites

Primary fieldwork took place at three non-contiguous sites (figure 1.2), heterogeneous in habitat structure and usage, two in England: Rishworth moore, West Yorkshire (N 53°38'29.61", W 1°58'24.24", elevation: 280m) and Dove Holes quarry, Derbyshire (N 53°17'59.17", W 1°52'37.47", elevation 338m) and one in Scotland: the Oa nature reserve (RSPB), Islay, Argyll and Bute (N 53°37'29.24", 6°7'22.08", elevation 93m). All three sites were baited with Niger seed *Guizotia abyssinica*, which provides a valuable supplementary food source for Twite and allows efficient capture for monitoring (see Conservation evidence; Raine 2004).

Rishworth moor, West Yorkshire, is a former millstone grit quarry in the dark peak region of the English Pennines, now owned by Yorkshire Water. It is surrounded on three sides by open, ovine grazed moorland which is dominated by Bracken *Pteridium* during the summer months and maintains a dried herbaceous under story during the autumn and winter. The site is flanked to the south by Booth wood reservoir running east/west below the Twite breeding colony of Oxygrains clough, beyond the reservoir to the south is the M60 motorway. This site caters for much of remaining English breeding population, with birds regularly moving 20-30km between satellite feeding stations and breeding sites in West Yorkshire and Lancashire.

Dove Holes, Derbyshire is a large site centred around an active aggregates quarry which sits at the boundary of the Peak District national park. Unlike Rishworth, 38.5km to the NNW, Dove Holes is dominated by high intensity, ovine grazed, limestone acidic grassland and heavy industry. Furthermore, Twite are primarily cliff nesting in these quarries (Frost 2008), and may have gradually increased in number over the past decade (Dunning et al. 2016). This site seems to be isolated from the highly connected breeding sites in Yorkshire and Lancashire despite apparent mixing over winter.

The Oa Nature reserve, Islay is the only non-breeding territory of the three primary field sites. The fields around the Kinnabus loch feeding station are managed by the RSPB specifically for Twite over Autumn/Winter, with a primarily fodder radish *Raphanus sativus* var. *oleiformis* seed mix. The surrounding area is managed as low intensity bovine (mixed commercial and traditional breed) grazed grassland, which opens to high coastal blanket bog. Sea cliffs to the North provide breeding territory for a small number of local Twite, however the winter flocks are composed of birds from Northern and Western Scotland, Wales and Ireland and make up ~14.2% of the total UK winter population (Wilkinson *et al.* 2018; D Wood pers. comm. 2017).

Secondary field sites

Secondary samples were collected by collaborators at non-contiguous sites in Sweden: Latnjajauren field station (N 68°21'36.32", E 18°28'59.7", elevation 981m), Mt Stor-Axhögen / Funäsdalen (N 62°46'24.88", E 12°32'4.95", elevation 1122m), Skändla (N 57°46'41.41", E 11°55'45.94", elevation 23m) and the Nidingen Field station (N 57°18'13.35", E 11°54'7.09", elevation 2m), Scotland: Machrihanish Bird Observatory (N 55°25'22.51", W 5°44'24.86", elevation 9.1m) and Wales: Connah's Quay Nature Reserve (N 53°13'23.05", W 3°3'47.55", elevation 5m). Of the Swedish sites, the two upland sites (Latnjajauren FS and Stor-Axhögen / Funäsdalen) represent breeding colonies where the lowland sites (Skändla and Nidingen Field Station) are non-breeding regions, and therefore the origins of un-ringed birds caught here are unclear. Both breeding sites sit on the boundary of the Arctic circle and are primarily mountainous and

sub-alpine in nature. Skändla is a region of Gothenburg, north of the metropolitan region and is absent of breeding Twite, likewise Nidingen field station is situated on an island south of Gothenburg and ~15km due west of Åsa, Sweden, it is a non-breeding and passage site for Twite.

Machrihanish Bird Observatory and Connah's quay nature reserve are both coastal non-breeding territories for Twite groups from the west of the United Kingdom, with some annual exchange of colour marked birds between the two. Machrihanish BO sits at the southwestern tip of the Mull of Kintyre with Twite coming to a strand of rocky shoreline and surrounding dune system. Connah's quay is an estuarine habitat in a region of otherwise heavy industry. Although Twite use the wider Dee estuary to forage during winter, they are attracted to brackish and freshwater pools on the nature reserve.



Figure 1.2. Map showing the location of primary and secondary field sites as described in text. The dotted line denotes the regions associated with the European subspecies, *pipilans* (UK and Ireland) and *flavirostris* (Continental Europe). The distribution raster shows the breeding/resident range (orange) and the non-breeding range (blue) courtesy of Birdlife international (2016).

Capture and monitoring of live birds

Twite captured were fitted with a BTO metal (Magnesium-Aluminium alloy) 'A' ring on the left tarsus, weighing 0.05g with a diameter of 2.3mm. In England, three plastic (or Celluloid) colour rings (AC Hughs, UK) with a diameter of 2.3mm, one above the metal ring and two on the right tarsus, were also fitted in a unique sequence to allow identification of individuals subsequent to ringing. On Islay, Scotland, a single black plastic (Poly(methyl 2-methacrylate), or PMMA) ring with a white engraved alpha/numeric code (Ecotone, Poland, diameter 2.7mm, weight ~0.015g) was fitted on the Right leg to allow for future field identification. Metal rings are fitted to all bird species captured for research under the BTO bird ringing scheme under license, colour marking was carried out under an additional BTO research license. Additionally, a wing cord measurement was taken as well as a weight and, age, sex and moult score as is standard BTO protocol.

DNA sampling

Non-invasive (without capture) or non-destructive sampling techniques are widely used to study the molecular ecology of threatened or elusive species without causing lasting harm to the animal (Taberlet et al. 1999; Negro and Kalma 2005). Samples were collected during the ringing process by either plucking a single contour feather from adults, undertaking a buccal swab in nestlings or the collection of dropped feathers. Twite were selected for sampling after capturing in a 'whoosh net' over a baited feeding station, based on their age, condition and/or breeding status. Although few Twite were sampled as nestlings, most birds are sampled as juveniles, post-fledging in nursery flocks near to the natal site. The mean distance juveniles were located from the natal site, 40 days post fledging was 1.4km (± 0.2), extending to 4.8km, (± 0.6) after 80 days (Raine et al. 2006b).

Ethical notes

Capture and metal ringing was carried out under a BTO bird ringing license. The addition of colour marks feather sampling and buccal swabs required further special measures permits issued by the BTO and on behalf of Natural England. No negative effects were noted as a consequence of any of the methods used during this study. None of the samples stored for genetic analysis required additional licensing, and all sequenced genetic and associated collection data have been made available on Gene Bank.

Software notation and statistical analysis

Word processing, figures and statistical analyses were conducted in R version 3.4.2, 'Short Summer' (R Core Team 2017) using the R studio graphical user interface, unless otherwise stated. Specific R packages were used for manipulation of EUring data (birdring, Korner-Nievergelt and Robinson 2014), tree building (ape, Paradis, Claude, and Strimmer 2004) and figures (GGplot2 as part of the tidyverse package, Wickham 2009). Additional open source packages were used in conjunction with the above and can be made available. Mean averages are given $\pm$ one standard error. Statistics associated with genetic analysis was carried out in DNAsp where stated (Rozas et al. 2017).

Mapping & GIS

Maps were built in R version 3.4.2, 'Short Summer' (R Core Team 2017) using the R studio graphical user interface, unless otherwise stated, with GGplot2 and the tidyverse package (Wickham 2009). Open source geoJSON files were accessed from geoJSON-maps and distribution layers from Bird Life International with permission (BirdLife International and Handbook Birds of the World 2016). Prior to mounting within the R console, distribution map data was split and converted to geoJSON files using QGIS (QGIS Development Team 2018).

Chapter 2

The spatial ecology of Twite groups in the Western Palearctic

Introduction

Understanding the spatial ecology of avian species is a prerequisite to understanding the forces which drive phylogeography (Irwin et al. 2011). The extent to which breeding groups mix throughout the non-breeding season has important consequences for the breeding ecology of a population (Finch et al. 2015), therefore, understanding spatial ecology is imperative to the effective conservation of a species. The study of migratory connectivity addresses the link between the breeding and non-breeding distributions of a species and the extent to which they mix over the non-breeding season (Webster et al. 2002; Norris, Wunder, and Boulet 2006; Andueza et al. 2014). These movement patterns affect the adaptation of species in relation to points in which populations mix and can therefore yield information about population structure, as well as spatio-temporal trends (Pain et al. 2004; Resano-Mayor et al. 2017). This is particularly apparent in species which show a high migratory connectivity; when those allopatric populations of a species winter in the same area but do not subsequently mix, and those which occupy a highly specialised niche (Pain et al. 2004; Andueza et al. 2014). Within the Western Palearctic the migratory ecology Twite has been intensively studied over several decades (Orford 1973; Sharrock 1976; Gibbons et al. 1993; Dierschke and Bairlein 2004; Raine et al. 2006a; McLoughlin et al. 2010; Balmer et al. 2013) but has not been considered as a whole since the Migration Atlas published in 2002 (Wernham et al. 2002).

The recent miniturisation of archival data loggers and geo-locators has allowed researchers to investigate how spatial ecology influences the life history of species in much greater detail (Bridge et al. 2011). However, these technologies do have limitations, which are particularly apparent when deployed on smaller birds such as the passerines (Bridge et al. 2013). Archival loggers require the capture of the animal for

data retrieval so are unsuitable for species with site high fidelity or low annual survival (Finch et al. 2017). The size of a bio-logging device and how it is mounted is also a limiting factor in smaller birds; a device which is too heavy or mounted incorrectly will likely have an adverse effect on the individual and may influence survival, perceived fitness and migratory ecology (Bridge et al. 2013; Bodey et al. 2018). These devices are also financially expensive and only follow a single individual, mass deployment requires a large investment in terms of both time and funding. These data can perhaps better be inferred through trends in ringing records (Macdonald et al. 2012; Andueza et al. 2014; Ambrosini et al. 2016), especially in smaller species. Mark/recapture studies are prone to biases and are rarely informative in terms of gene flow; long distance dispersal is difficult to observe and the method fails to detect stochastic events which affect dispersal (such as storms or climatic change), furthermore, where long-distance dispersal is detected, mark/recapture does not provide proof of subsequent successful breeding (and inferred gene flow) (Whitlock and McCauley 1999). These relationships can be better quantified when demography data is used in conjunction with complementary phylogenetic analysis. However, for the study of many species, marking and subsequent recovery of individuals remains the best source of information about their migratory ecology and life history (Reichlin et al. 2009). In contrast to bio-logging, mark-recapture techniques are spatially precise but temporally coarse (Finch et al. 2017), often only providing two-points per individual, but are much more affordable and can be deployed on a population scale. Ring recoveries also allow exploration of the causes of mortality and inexperienced juvenile migration strategies (e.g. McCulloch and Tucker 1992; Finch et al. 2017).

Methods

EURING Data

To study the movements within the Western Palearctic, Twite were colour-marked between 2013-2018. As well as this, data was collated from all available ringing and monitoring schemes within Europe. Data from the EURING Data Bank (EDB) was accessed.

For species with long distances between the breeding and non-breeding ground, ring recoveries are considered to be migratory movements if they exceed 100km from the breeding grounds, to accommodate for short-distance movements during the pre-migratory period or post breeding dispersal (Reichlin et al. 2009; Finch et al. 2017). Because some small constituent of the European Twite population is resident during the non-breeding season a lower cut-off point of 10km was chosen to exclude dispersal. Where the analysis concerned phenology, recoveries were defined to one of three seasons following Finch et. al. (2017), but tailored to the annual cycle of Twite using EURING ringing data, as well as breeding phenology accounts published previously from within the region; the autumn post-breeding migratory period (from September up to and including October), the non-breeding season (November up to and including March) and the breeding season (April up to and including August). Duplicate ring numbers were excluded from the data, this corrected for high site fidelity and observer biases which caused several birds to be counted twice in the recovery analysis.

Condition of recovery was extracted from the EURING data; of those recovered dead, the circumstance of recovery was also acquired in order to explore any factors which may be effecting Twite during the annual cycle or within specific regions within their range. Recovery points were then broken down into subsets by the country and season ringed, these points were plotted and the breeding and non-breeding regions defined using a 75% kernel-density plot for the continental and British groups. A key question which this project sought to address is connectivity and subsequent gene-flow between the British and the continental breeding groups of Twite and so, these ringing and

recovery data were plotted by ringing origin in order to show the numbers which have moved across various land and sea borders. All maps and figures are annotated with the sample size (n), if not detailed in the figure legend. Countries are referred to using two letter EURING codes (for example, GB denotes Great Britain), a key to which can be found in table 2.1. Ringing effort over time has been visualised in order to contextualise temporal disparities between the British and continental breeding centres, for this all records (e.g. newly ringed birds, recaptures and recoveries) are included.

Offshore records

A number of offshore records exist within these data. These records, although sparse, can provide information into the migratory routes that Twite follow between breeding and non-breeding territory, and perhaps suggest connectivity between groups. The propensity for sea-crossing is of particular importance here, as for connectivity to exist between the British and European breeding groups Twite will have to cross either the English Channel or the North Sea. The number of birds which die at sea unobserved is impossible to quantify, but the occurrence of species within data from shipping and offshore infrastructure can suggest a capacity for sea crossing and indeed the regularity at which such crossings may be undertaken within species.

Ship assisted records

Two Twite in the EURING database were recovered on ships. Because this is suggestive of an anthropocentric bias to movement, these data have been omitted from the analysis. However, in the context of offshore movements they are included in the map showing reported location of Twite at offshore infrastructure (figure 2.12).

Migratory connectivity

Following Ambrosini et al. (2009), orthodromic distances (the shortest possible path taken between two points across the surface of the Earth; see figure 2.1) were calculated from geographical coordinates (Long, Lat) of Twite records from both the breeding and non-breeding seasons. Distance Mantel tests of correlation were carried out between pairwise distance matrices, which represent the spatio-temporal relationship between two birds, of breeding sites and non-breeding sites at three scales: within the British group, within the continental group and across the European population as a whole. Positive correlations from previous analysis indicate that birds that breed close together also spend the non-breeding season close together (Ambrosini et al. 2009; Finch et al. 2017a). Seasons were defined specifically for this analysis because few birds have been ringed on the Scandinavian breeding grounds during the breeding season and subsequently recovered during the non-breeding season; Here the breeding season is defined as May-July and the non-breeding season as November-February. These narrow definitions also help to confirm breeding status (rather than passage or non-breeding), the assumption is that those caught during this period are on the breeding grounds and that any abnormal noise from stochastic movements or status are corrected for by the quantity of breeding individuals. To further boost sample sizes, birds ringed on the winter grounds which returned to breeding sites have been inversed and these data are included. Birds which qualify under these criteria were mapped, see figure 2.10.

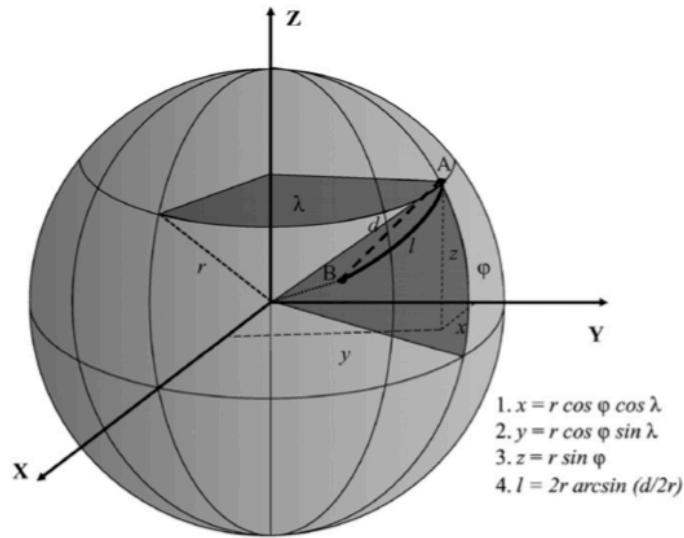


Figure 2.1. Orthodromic distances are calculated in order to convert frame distance between ringing and recovery as stored in EDB (d) to account for the curvature of the earth (A,B). Longitude and latitude are converted into three dimensions (x,y,z) with equations (1,2 & 3). r conveys the average radius of the earth. Euclidean distance is calculated with Pythagoras's theorem and finally converted into orthodromic distance (l) with equation 4. Reproduced from Ambrosini et al. (2009).

Results

Demography

Of the 22 622 Twite ringing records submitted to EUrling data bank, 13,906 rows represent new birds ringed, leaving 8,716 as subsequent recaptures. Of those new birds, 61% ($n = 12\,252$) were sexed on plumage or behaviour at the point of ringing; 41.2% ($n = 5740$) were sexed as female and 46.8% ($n = 6512$) as male, leaving the remaining 11% undetermined. Sex ratio varied by site, but was most extreme in the contemporary data from Dove Holes, Derbyshire between 2015-2017 where males outnumbered females by as much as 3:1.

Age codes were recorded for all Twite ringed. 0.8% ($n = 123$) were aged as nestlings. Brood sizes of nests visited had a mean of 4.735 (± 1.023) with 5 being the most frequently encountered. The majority of Twite were captured post fledging. Age codes 2 & 3 represent juvenile birds, here accounting for 54% ($n = 7514$) of birds aged, the remaining birds were aged as codes 4, 5 & 6, which all represent adult, in this case > 1 calendar year Twite.

Most birds were encountered during the winter months with 67.3% Twite ringed within this period. Breeding takes place between May and August and in Europe, and accounts for 10% of ringing records ($n = 1507$). The remaining months are spent as an intermediate or passage period and equates to 21.8% of records ($n = 3037$). Across the data sex of individual did not predict migratory distance ($t = 0.97$, $df = 14734$, $p = 0.3$), nor did age ($t = 6.47$, $n = 3841.5$, $p = 1$).

Ringling effort

Twite ringing records have been submitted to the EUrling data bank since 1937 and up to the most recent data submission at the time of access (March 2018), 2017. However, the number of birds and regions where Twite have been ringed and subsequently submitted to the EDB, has fluctuated over time. Although low numbers (< 5) have been submitted annually in almost all of the countries where ringing has taken place since at least the 1970's, a disproportionate amount of effort has been put in where specific projects have required, see Dierschke (2001) Raine (2006) & McLoughlin (2009). Ringing effort is therefore quantified as the number of Twite processed, by region, over time (figure 2.2). British effort did not surpass > 50 bird until 1999 (from one record submitted in 1998 to 152 in 1999) and peaked in 2010 ($n = 1853$); a response to increased monitoring (see Wilkinson et al. 2018). The combined German centres of Hiddensee (DEH), Radolfzell (DER) and Wilhelmshaven (Heligoland, DEW) contributed the majority of continental ringing effort (> 50) between 1958 ($n = 57$) and 1971 ($n = 69$) and peaked in 1965 ($n = 1120$).

Although the EDB has records of Twite from 18 European countries and the North Sea (see figure 2.12), Twite records were only submitted from 12 of these. Twite were ringed primarily as a breeding species in only four countries (the United Kingdom, Ireland, Norway and Sweden) and a non-breeding or passage species in the remaining eight.

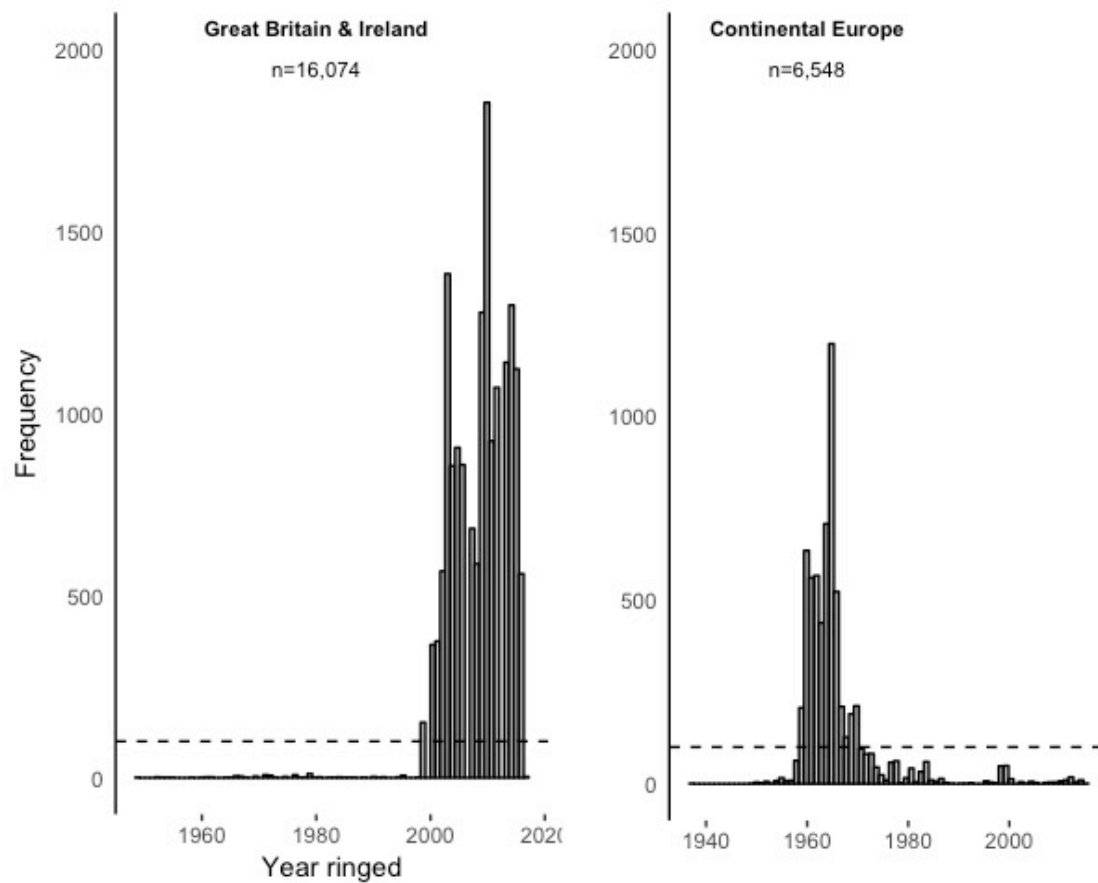


Figure 2.2. Twite ringing effort over time and by region, these data include all birds captured for ringing and monitoring. Where much of the British effort took place after 2000, continental effort has tailed off since the 1980s. The horizontal dotted line denotes 100 birds processed.

Table 2.1 The number of Twite ringed in each region represented in the EDB data. Areas which have a 0 have either reported only a recovery of a ringed bird, or have been reported as the area of recovery by the original ringing scheme. This is not to say that Twite have not been ringed within these regions, just that these data have not been made available through the EDB.

Country code	Region	Notes	Ringed here (n)
-B 00	North Sea	00, At sea, unspecified.	0
AU	Austria	Listed in data as CS.	0
BL	Belgium		259
CZ	Czech Republic		17
DE	Germany	Including DF & DD.	4,815
DK	Denmark		89
ER	Ireland		451
FR	France		3
GB	United Kingdom		15,623
HG	Hungary		0
IA	Italy		0
NL	Netherlands		1,095
NO	Norway		187
PL	Poland		0
RO	Romania		0
RS	Serbia	Formally Yugoslavia thus listed as YU.	0
RU	Russian Federation	Including SU	0
SF	Finland		8
SK	Slovakia		14
SV	Sweden		61

Recovery circumstance

Although ~97% of all Twite represented within the EDB data were recovered alive, 2.2% were recovered dead, sick or moribund. No data was recorded for the remaining 0.8% of birds. Of the 488 that were recovered dead, circumstance of recovery was recorded. Circumstance of recovery presumes the outcome is mortality where the circumstance is not specified as deliberate capture by a bird ringer. The EDB has 39 circumstance categories listed for Twite, five of which are deliberate capture methods for monitoring and presumes that the bird was subsequently released, the remaining 34 are presumed to be causes of mortality. These have been combined into five categories; environmental which includes climatic or seasonal factors such as food shortages, storms and cold weather; human, intentional, which includes deliberate capture for non-monitoring such as hunting or taken for caged/aviary stock; human, unintentional, are those records which would have otherwise survived were it not for passive anthropocentric factors such as collisions with windows, power-lines and traffic; predation are those records which were recovered having been discarded following predation by another animal and unknown which are those records without specified recovery circumstances. The data shows that both predation and unintentional human factors are the highest cause of mortality in these data, indeed, 36 individuals were recovered after collision with a vehicle and 42 were taken by domestic cats, for comparison only 13 were taken by predatory birds and only one was recorded as taken by a wild mammal. 113 were deliberately hunted for food or sport. Although low numbers, when domestic cats are accounted for, the largest recorded cause of mortality to Twite in Europe is unintentional persecution from anthropocentric factors, see figure 2.3.

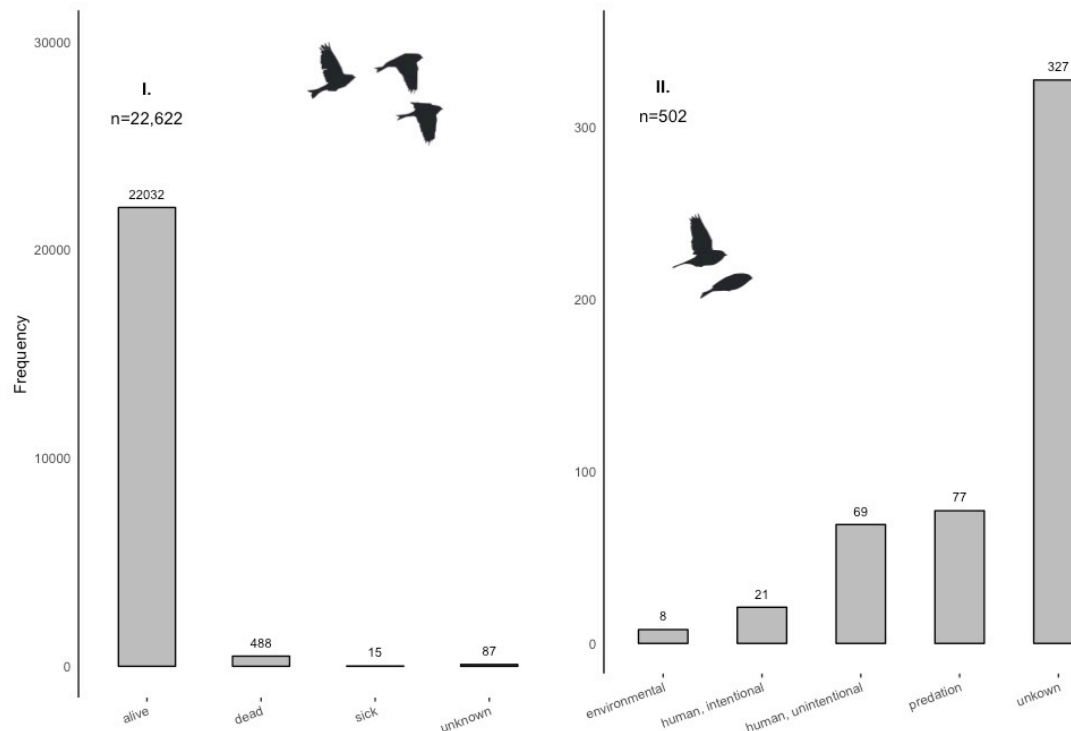


Figure 2.3 Condition (I) and circumstance (II) of Twite recoveries from the EDB. Note that unintentional human activity is the greatest threat amongst known causes of mortality, followed by predation, which here includes that of domestic cats.

Recovery locations

In order to ascertain connectivity between the two major European breeding centres, recoveries have been assigned to a breeding group; These are Great Britain and Ireland and continental Europe. When ring recovery data is plotted for the continental group their defined migratory ecology becomes clear (figure 2.4). These birds undertake a dramatic shift towards lower latitudes annually during the colder winter months. Mean latitude was plotted for each month of the year alongside a 75% kernel density plot to depict seasonal spatial ecology between the breeding and non-breeding season.

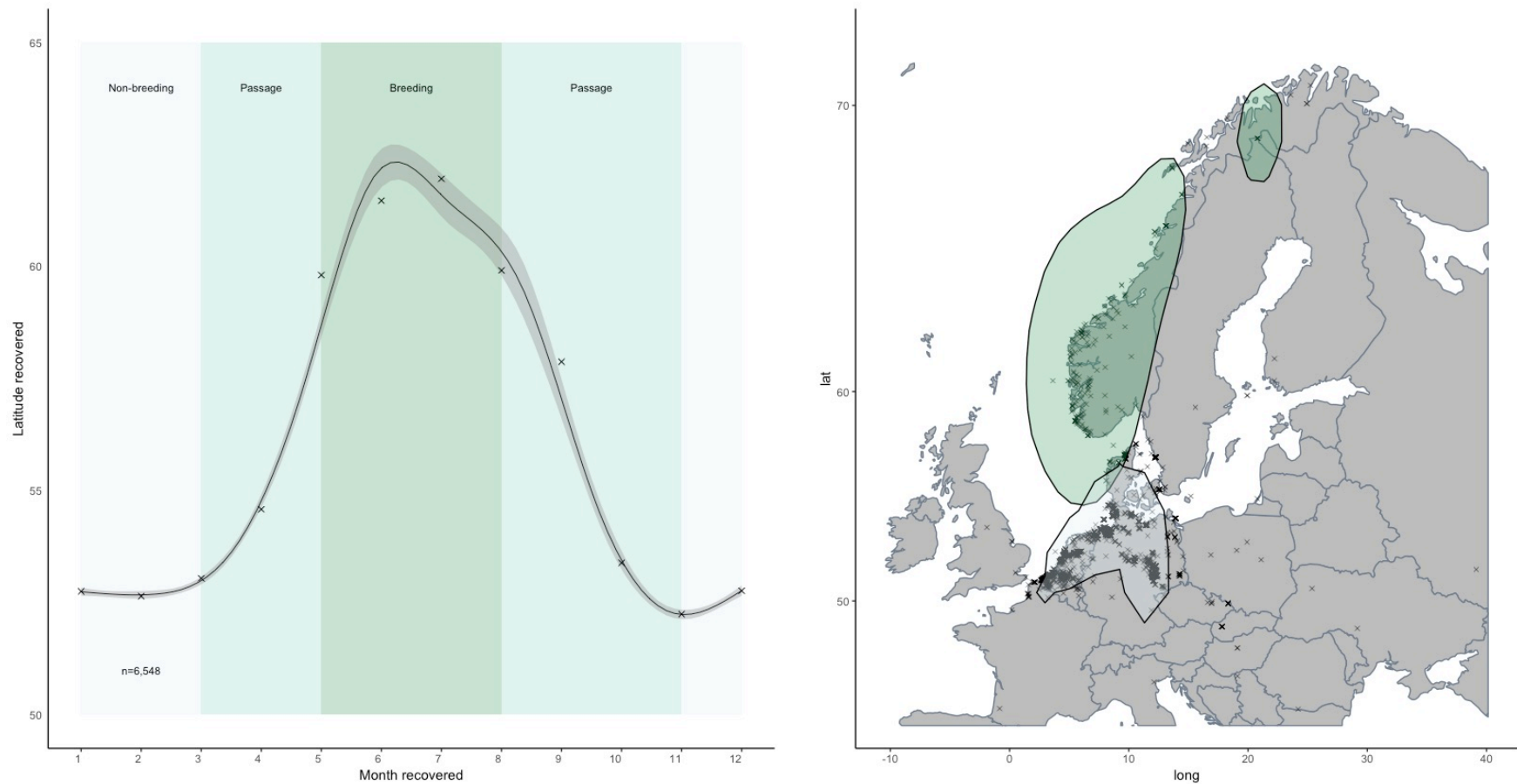


Figure 2.4 Mean latitude (x) of Twite over month within the continental group (right), alongside 75% KD projection (left); The green polygon denotes the breeding and white the non-breeding period.

When the same analysis is applied to the British and Irish group (figure 2.5), the picture is convoluted by the apparently differing migratory ecologies of this fragmented population; Whilst Twite from the northern Scottish Highlands and Fair Isle move down the east coast of Scotland, those breeding groups along the Atlantic west coast in Wales, Northern Ireland and the Scottish Hebrides winter between the Scottish west coast and the Welsh Dee estuary. The English breeding groups in the Pennine hills winter between East Yorkshire and Kent on the English south east coast with the highest densities between Norfolk and Suffolk. Unlike the continental group, a number of birds within this region remain in the breeding grounds over the non-breeding period.

The number of Twite recovered on the English breeding grounds over winter has caused the primary non-breeding range on the south-east coast to fall outside of the kernel density plot. This highlights a bias towards where these birds are ringed, rather than recovered, for a better representation see figure 2.9.

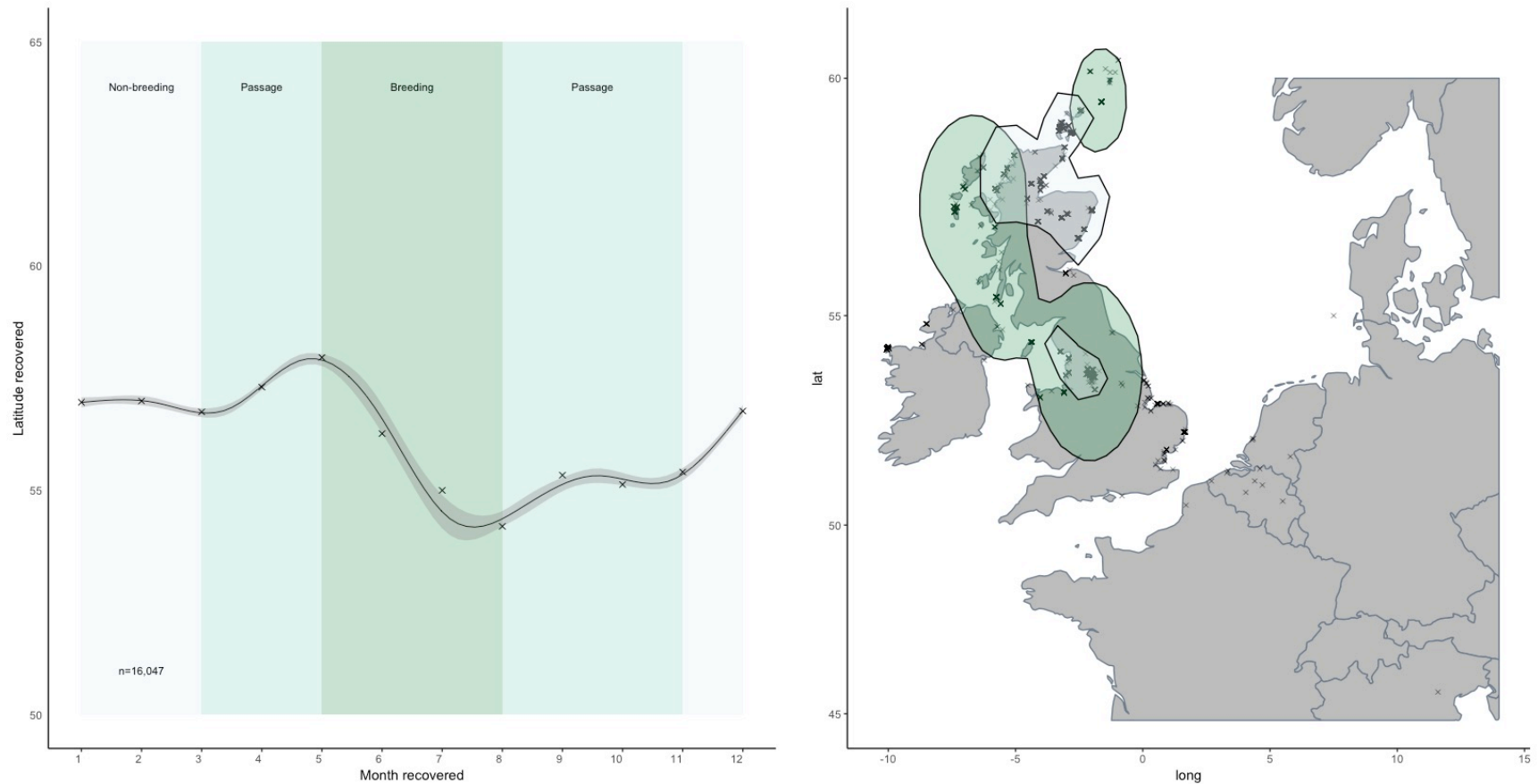


Figure 2.5 Mean latitude (x) of Twite over month within the British group(s) (right), alongside 75% KD projection (left); The green polygon denotes the breeding and white the non-breeding period.

Recoveries from each group reflect these non-breeding distributions. According to the EDB data, 16 out of 8,340 (0.1%) Twite ringed in Great Britain have been recovered in five continental European countries; eight of which were on the west coast of Belgium, a further four were recovered on the west coast of the Netherlands, and a single bird on the northern French coast. One British Twite was recovered in the Republic of Ireland and another, perhaps most surprisingly, in the Italian Alps (outside of the Twites continental non-breeding distribution).

A single Twite ringed in the Republic of Ireland has been recovered in Great Britain. These data also include five birds ringed in continental Europe and recovered within the UK, all of which are from the Netherlands during the non-breeding season. The continental group however has a much greater connectivity between regions, these birds move from the Scandinavian breeding grounds and spread out widely into western Europe and the continental interior; 108 of 166 (65%) of Norwegian ringed Twite records submitted to the EDB have been recovered in 13 regions of continental Europe, 49 of which represent recoveries from the three German recording regions (DD, DE, DF). Of Twite ringed during the non-breeding season across six continental European countries, 107 (2%) were subsequently recovered in Norway during the breeding season. Birds ringed in these regions have also been recovered during subsequent years during the non-breeding seasons in other countries; of birds, ringed in Netherlands and submitted to the EDB, 251 (23%) have been recovered in Germany and 202 (19%) in Belgium. Moreover, 158 (4%) birds ringed in the German recording area have been subsequently recovered in the Netherlands and 96 (2%) in Belgium, suggesting high vagility over winter months along the North Sea coast

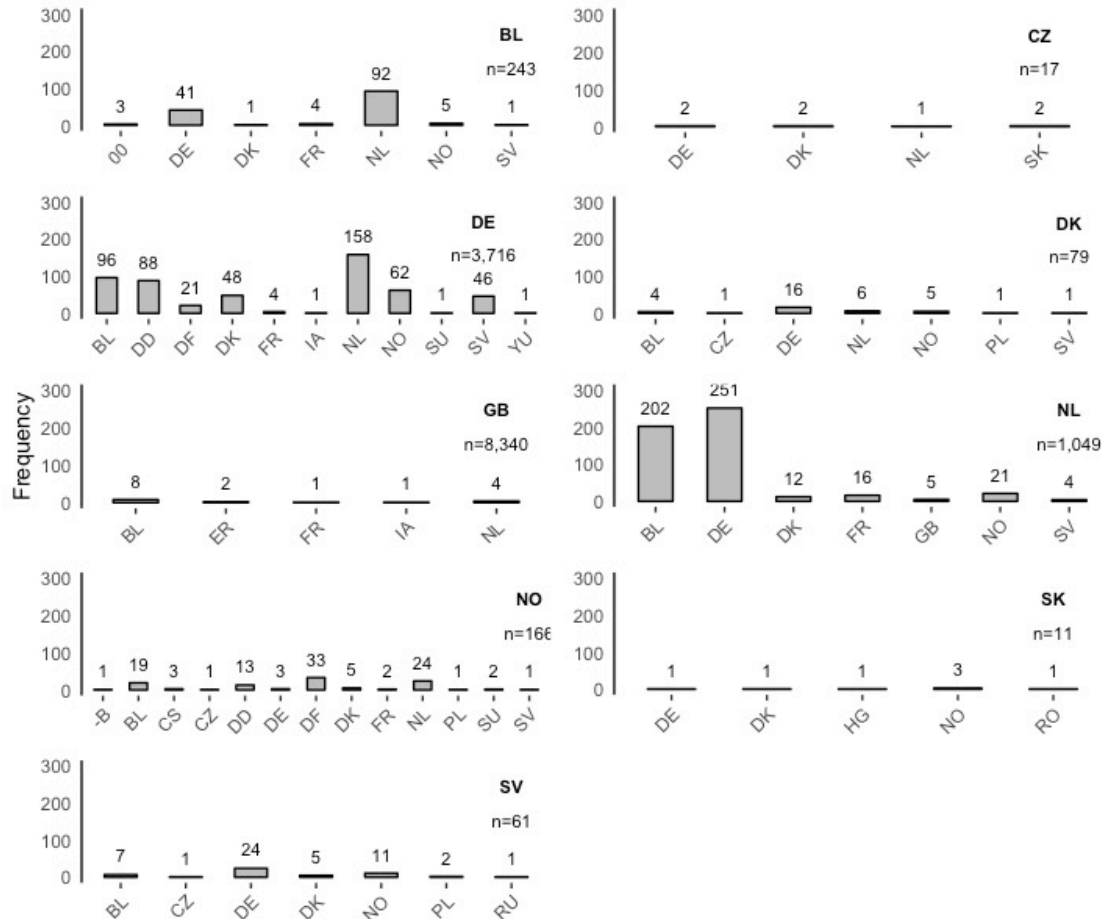


Figure 2.6 The number of records submitted to the EDB by country. Frequency plots show the number of birds recovered and location of recovery by region.

In order to address the spatio-temporal distribution of Twite recoveries from the British breeding group these data have been sorted into month recovered, figure 2.7. These data find Twite are more likely to show stochastic movements during passage periods and over winter. Spring records from January and March and a winter record from October show British Twite crossing the North Sea to areas of continental Europe immediately adjacent to the English non-breeding range. These are thus consistent with birds wandering from, or 'over-shooting' well-established winter territories. Also, apparent from these data is the seasonal movements between the inland breeding and coastal non-breeding ranges, however even during the winter months some constituents of each population stay within the breeding grounds.

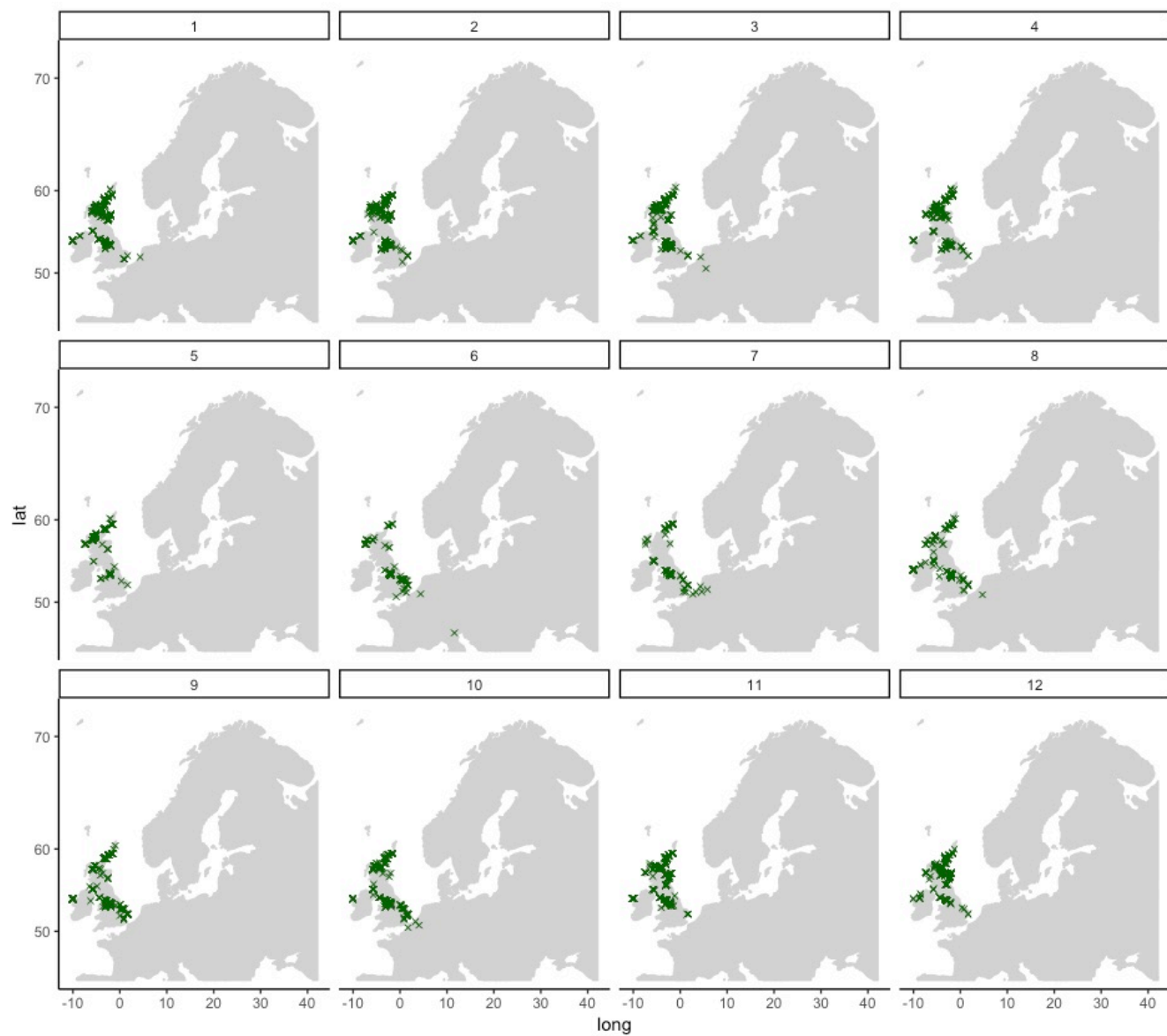


Figure 2.7 Recoveries of British ringed Twite broken down by month and location recovered.

Within the continental data the difference between the breeding and non-breeding distributions are more marked; However, much like the British and Irish group, Twite are much more likely to wander during the passage and winter months, here with recoveries on the British mainland during October and November. Twite begin to arrive in central Europe from the Scandinavia and peak during November, however they are apparent in some number until April, when they move back North to the Scandinavian breeding grounds during May. The few records between May and August represent the breeding season where Twite are on the Scandinavian breeding grounds. During the Autumn passage, there is an increase in recoveries in the European interior as far south and east as Romanian and Russia as birds over-shoot wintering grounds further north. The direction of winter recovery ($^{\circ}$) was highly significantly different between a random sample of 1000 birds from both the British (mean 168.7°) and continental groups (mean 205°) (t-test; $t = -9.0089$, $df = 1998$, $p = >0.0001$). No relationship was found between age class and the variance of recovery distance between a random sample of 100 adults and juveniles for the British group; (F-test; $F = 0.6$, $df = 99,99$, $p = 0.03$), the continental group lacked sufficient data to directly compare, with 130 juvenile birds and only 22 adults. Twite from the continental breeding group were also recovered significantly further (mean = 553.2 ± 326.7) from their ringing origin site than those from the British breeding group (mean = 201.1 ± 60) (t-test; $t = -11.981$, $df = 1153$, $p = >0.0001$).

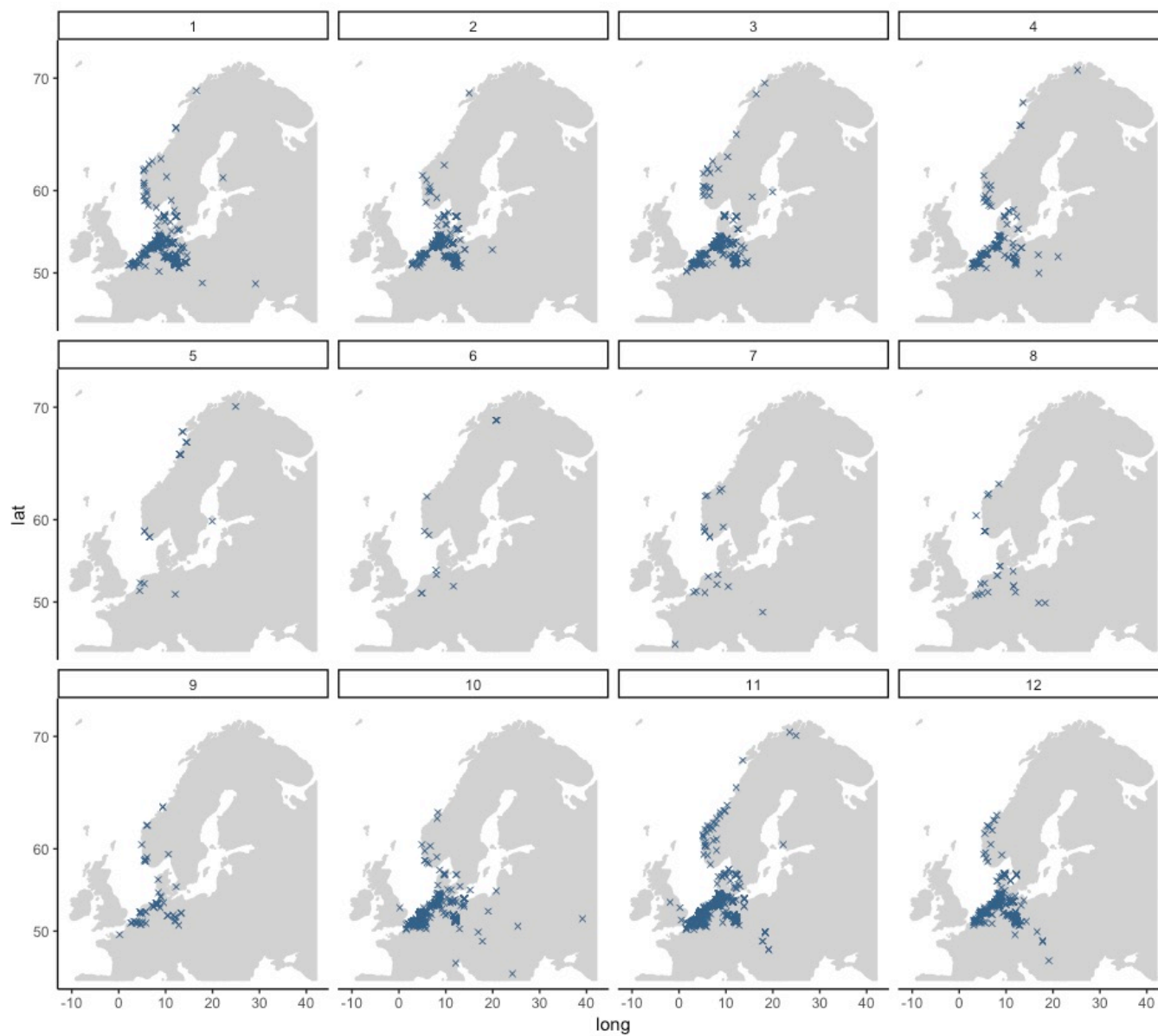


Figure 2.8 Recoveries of continental ringed Twite broken down by month and location recovered.

Within England, ~700 Twite have been ringed as part of a dedicated monitoring project since 2013. Despite maintaining isolation over the breeding season, the Pennine and Derbyshire breeding groups mix freely in the same regions of South East England during the non-breeding season, see figure 2.9. However, on returning to the breeding grounds, although Pennine Twite have used the Derbyshire breeding grounds as a stop-over site, they are yet to be recorded breeding in the area, no birds from the Derbyshire population have been recorded further north than their restricted breeding range. Of 71 Pennine and 23 Derbyshire ringed Twite, recovered during the non-breeding season, 75% kernel density plots reveal that the non-breeding range is synonymous between the two groups.

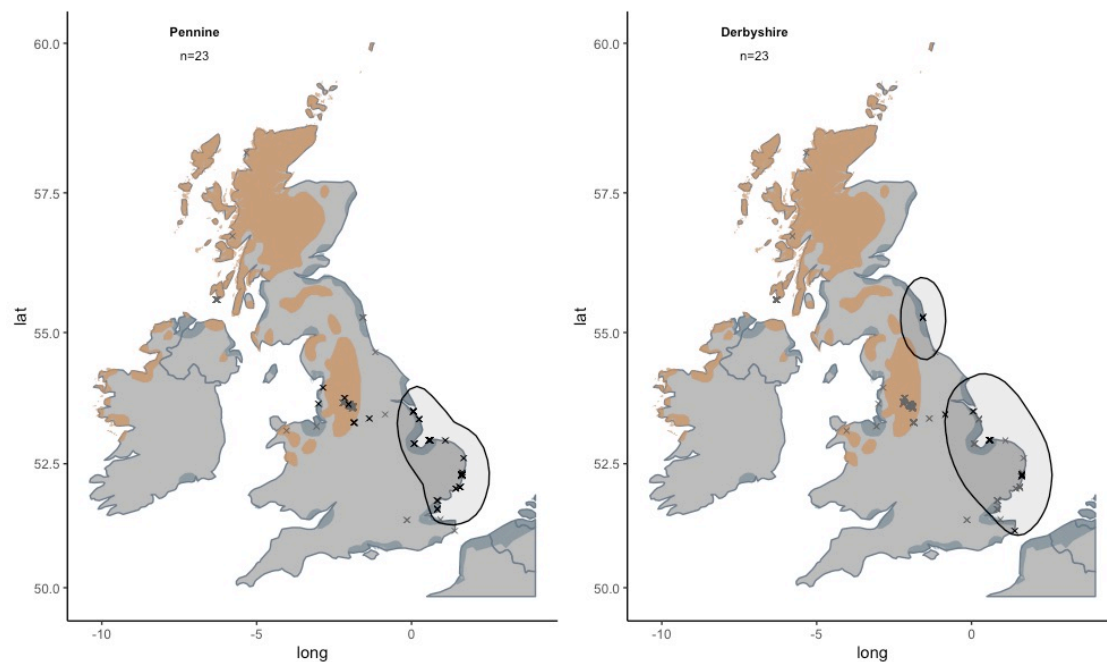


Figure 2.9 75% KD projects for recoveries from two isolated English breeding groups which share wintering grounds.

Migratory connectivity

There is strong connectivity within the UK ($r = 0.52$, $n = 242$, $p = 0.001$), this is consequential of the English groups wintering exclusively in the south east where Scottish, Welsh and Irish birds winter on the north-east, west and north-west coasts. Conversely, the continental group winter across a much larger area and as a result have a weak migratory connectivity ($r = 0.07$, $n = 81$, $p = 0.18$). Together, the European population as a whole has a strong migratory connectivity ($r = 0.68$, $n = 323$, $p = 0.001$).

Individuals were only selected for the analysis which have been ringed in either the breeding or non-breeding season and have been subsequently recovered in the subsequent breeding or non-breeding season. For a full selection criteria see methods: migratory connectivity, see figure 2.10 & 2.10 for a map of these movements and raw data.

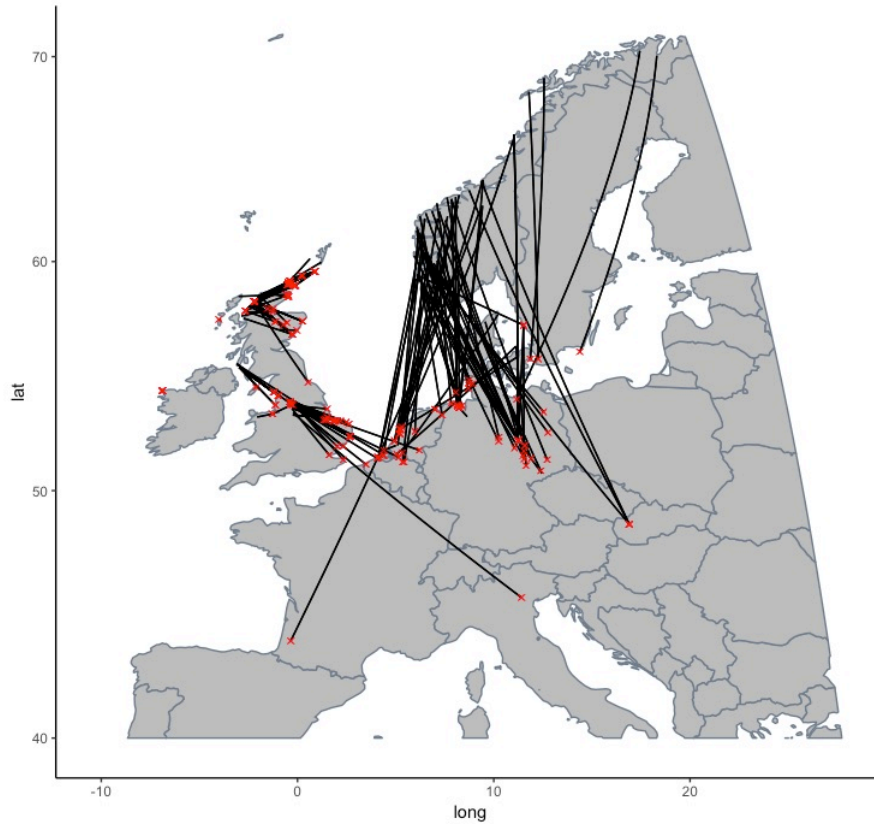


Figure 2.10 Orthodromic distances between the breeding and non-breeding locations of Twite used in the analysis of migratory connectivity.

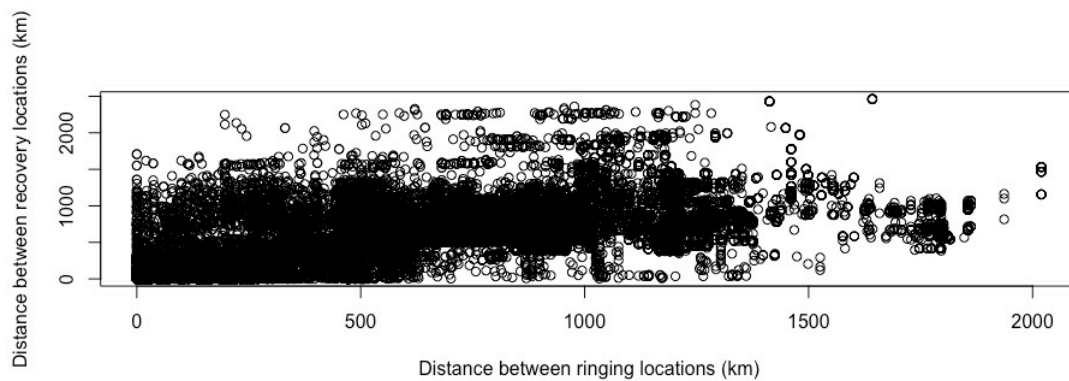


Figure 2.11 Distances between pairwise ringing and recovery locations of Twite in Europe used to analyse migratory connectivity.

NSBC data

North Sea Bird Club data contained only 35 records of Twite representing 117 animals between 1979-2014 (36 years). This is lower than other Scandinavian/Feno-Scandinavian migrants for which data was accessed (figure 2.13; 2.14). These data show that of the carduelinae finches sampled: Twite (2.13.A) , Linnet (2.13.B), Goldfinch (1.13.C) and Siskin (2.14.B), only the Siskin is recorded regularly on offshore infrastructure. This is corroborated by ringing records showing that siskin regularly cross the North Sea between the UK and Scandinavia whilst maintaining stable social bonds (Senar *et al.* 2015). Both of the Fringillinae species native to the region, the Common chaffinch (2.14.C) and Brambling (2.14.D) and the Common starling (2.14.A), a widespread breeding species in northern and western Europe (Newson *et al.* 2008; Musgrove *et al.* 2013), are known to migrate from Scandinavia to accommodate frugivorous winter dietary requirements (Newton 1967; Newton 2006). These migratory habits are reflected in the NSBC data; both the chaffinch and the brambling show similar peaks in occurrence in 1992, 1998 & 2000 which corresponds to their eruptive non-breeding distribution model. The Starling is more ubiquitous in occurrence throughout these data and likely represents birds moving away from harsh winter conditions in Russia and Scandinavian as part of their annual cycle (Feare 1996).

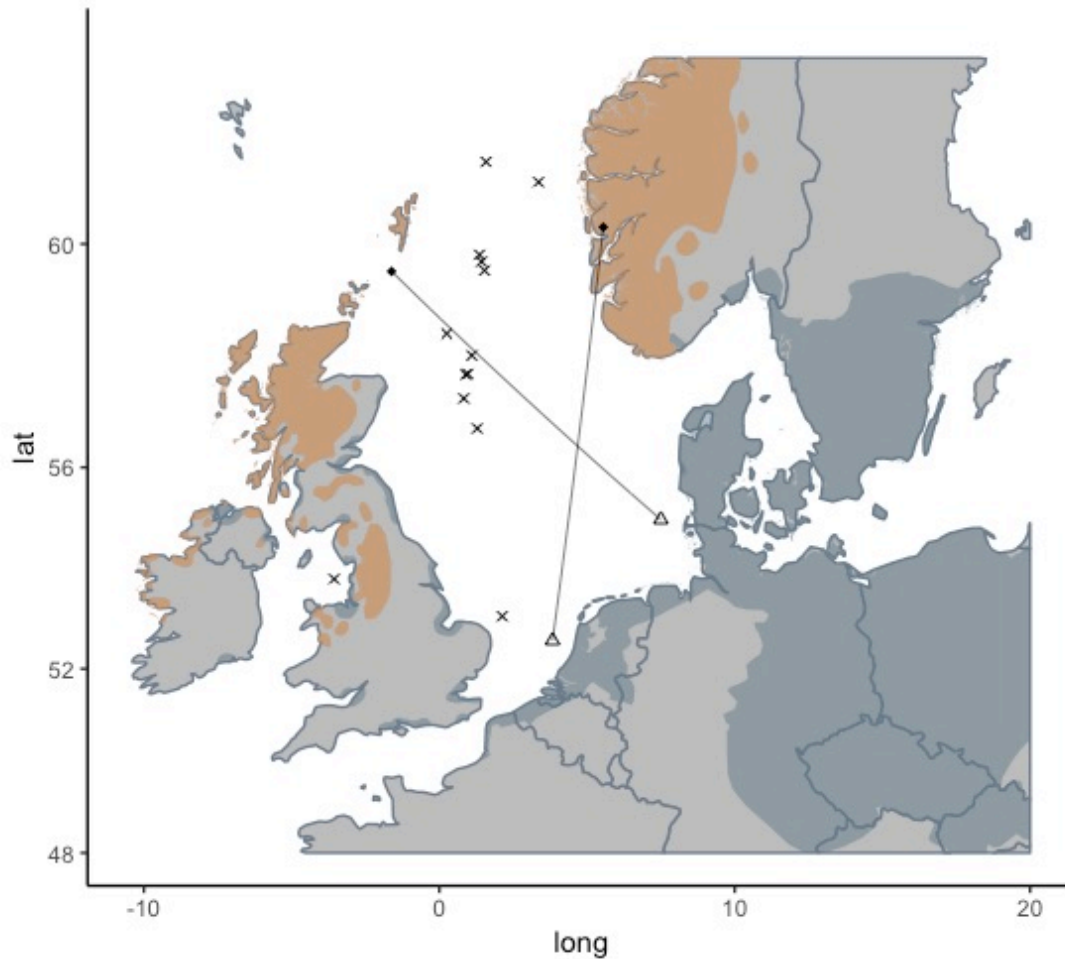


Figure 2.12 Locations of putative Twite sightings on offshore infrastructure depicted as X, as recorded by the North Sea Bird Club, 1979-2014. Also shown are two ship assisted records omitted from Euring data analysis, where ringing origin is a dark point and point of subsequent recovery on ship, a triangle. Areas of tan depict breeding, and blue the non-breeding range.

Table 2.2 Species for which data was accessed from NSBC, showing mean number recorded on offshore infrastructure annually as well as the standard deviation ($\pm$).

Species	Years	Mean	SD ($\pm$)
Brambling	36	454	700
Chaffinch	36	1184	3666
Goldfinch	36	5.28	17.9
Greenfinch	36	14	15.6
Linnet	36	4.33	10.2
Siskin	36	25.9	45.1
Starling	36	6357	7576
Twite	36	3.25	10.8

The mean number of Twite reported annually offshore was 3.24 ($\pm$ 10.8), the lowest of the eight species addressed in this study. However, the closely related Linnet is also poorly represented in offshore data with a mean of 4.33 ($\pm$ 10.2) reported annually. The distribution of Twite records from these data is centred around three regions; the Forties and Viking shipping regions off the east and north-east coast of Scotland respectively and the English inshore waters (within six nautical miles) off the Norfolk and Lancashire coasts. Two records from the northern edge of the Viking shipping region correspond to the continental breeding range (figure 1.2). Of these records, most (10) occurred during April and September (eight), both key passage months for Twite, with no records submitted between June-August which may suggest a reduction in migratory or wandering behaviour during the breeding season. Where weather was recorded (in 15 cases), wind ranged from Beaufort scale two to gale force eight, there is not enough data to determine the affect this may have on bird movements.

Although the records from six installations in the Forties and Viking shipping regions could not be verified (Fig 2.12.), records of Twite have been confirmed from Morecambe bay, England (a freshly dead individual), and the Mearsk Curlew (210km east of Aberdeen). Another record off the north Norfolk coast, England is likely to be reliable based on proximity to the non-breeding range. A single Twite was also recovered dead from the FINO 1 offshore platform 85km WSW of Heligoland island (54°01'N, 6°35'E) in the North Sea during an autumn survey of avian collisions [Huppopp et al. (2016); supplementary information] and not included in NSBC data.

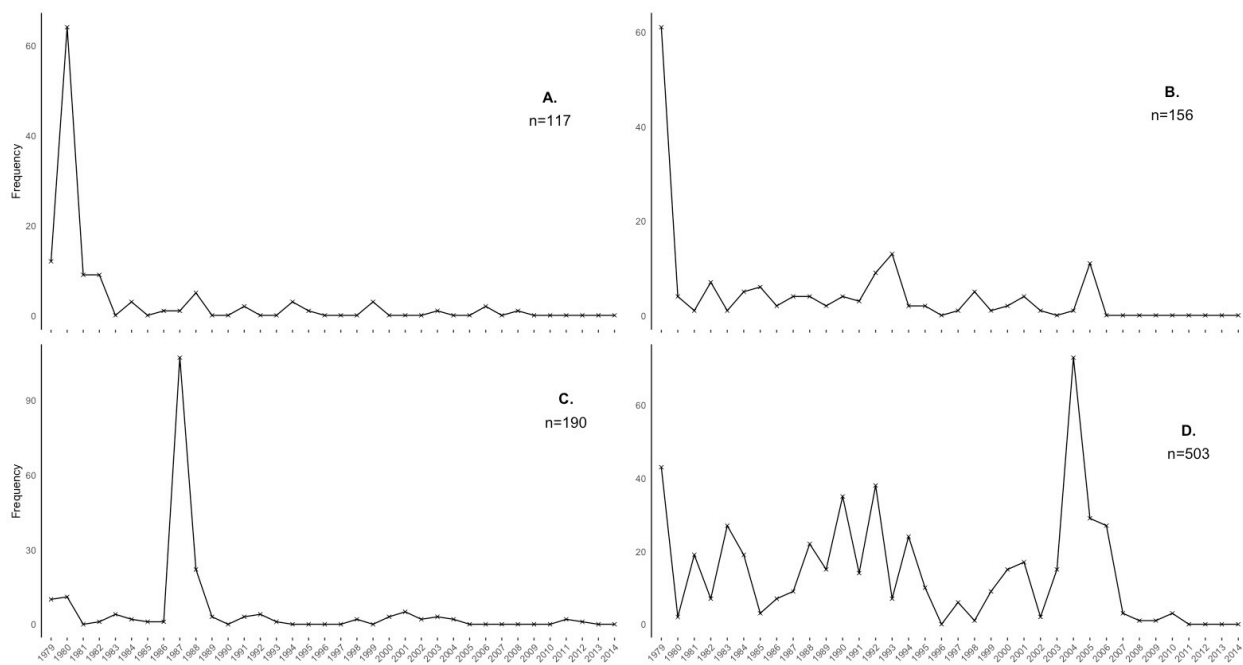


Figure 2.13. The frequency of four species recorded on offshore infrastructure between 1979-2014. Here, Twite, $n = 117$ (A.), Linnet, $n = 156$ (B.), and Goldfinch, $n = 190$ (C.) are scarce records where Greenfinch, $n = 503$ (D.) is more frequently encountered.

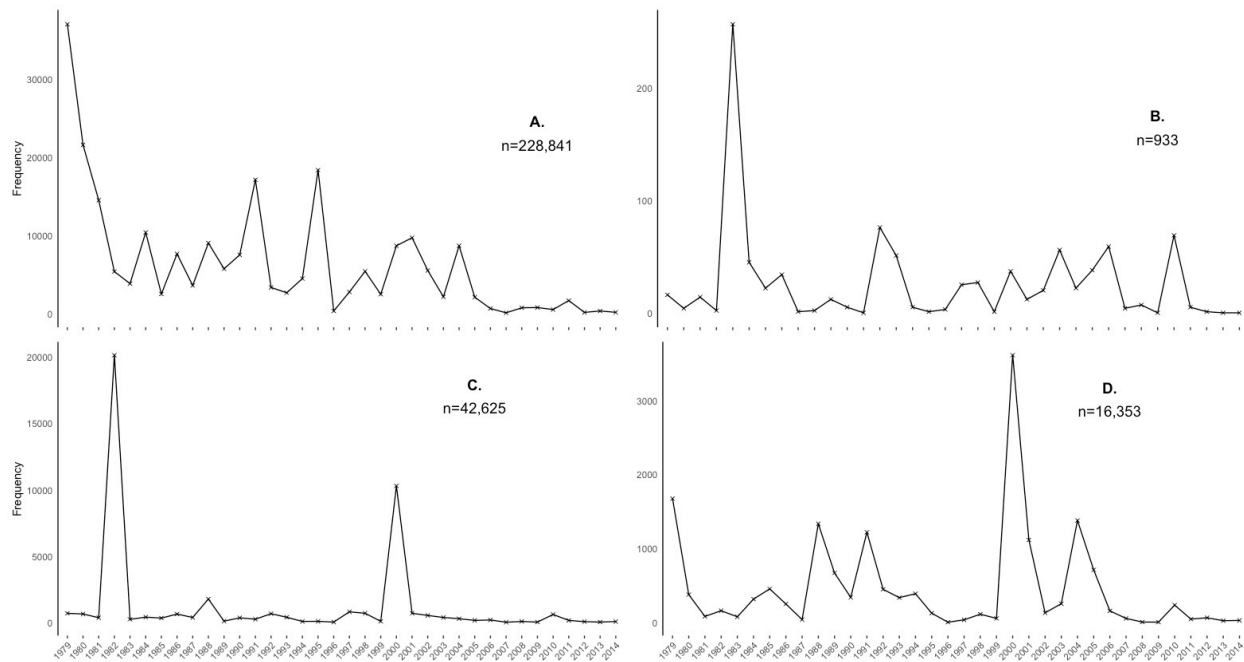


Figure 2.14. The frequency of four regular Anglo/Scandinavian migrant species recorded on offshore infrastructure between 1979-2014. Here, Common Starling, $n = 228\,841$ (A.), Eurasian siskin, $n = 933$ (B.), Common chaffinch, $n = 42\,625$ (C.) and Brambling, $n=16\,353$ (D.) are all encountered annually. The Chaffinch and Brambling however have a more eruptive migration model.

Discussion

Here, I present the first European-wide analysis of Twite ringing/recovery data since the Migration atlas (Wernham et al. 2002) and, address spatio-temporal relationships and connectivity between the two major western European breeding groups. These data are consequential of several long-term studies on Twite within Europe, see (Dierschke 2001; Raine 2006; McLoughlin 2009; Wilkinson et al. 2018), and are therefore temporally mismatched. However, I have supplemented data accessed from the EDB with 1612 records of modern (2013 - 2018) ringing/recovery data from England and records of occurrence from the NSBC. Together this analysis has facilitated the identification of migratory routes, connectivity and points of contact between the two major breeding groups of Twite in Europe.

Migratory ecology

Across these data the sex of an individual did not predict migratory distance, nor did age. Continental Twite appear to have low (or weak) migratory connectivity, meaning individuals spread over a large area during the non-breeding season and freely mix with individuals from other breeding regions. British Twite have a high (or strong) migratory connectivity, with individuals from differing breeding groups, in this case England, Wales, Western Scotland and the Northern Isles, using discrete, population specific non-breeding areas. The continental group has a smaller sample size of birds which qualify under the criteria for inferring migratory connectivity (see methods) ($n = 81$ to the British $n = 242$), and therefore continental groups could have a stronger migratory connectivity than predicted here. These findings are however predicted by analysis of other long-distance migrants which are often associated with weak connectivity (Finch, Butler, et al. 2017) and supported by the ringing/recovery results presented by this study.

Although the continental breeding range (>1000km), is wide relative to British Twite, there is no detectable connectivity or clustering of records during the non-breeding season. Whereas, within the UK where Twite groups breed in closer proximity (within ~500km of each other), they show strong connectivity with non-breeding records clustering strongly in discrete locations. The apparent lack of connectivity within the continental non-breeding season suggests that location of recovery is dependent on a series of fine-scale factors, which vary annually and therefore confound apparent connectivity (Finch, Butler, et al. 2017). Furthermore, rapid climatic change has allowed many species to winter at more northerly latitudes (Gregory et al. 2009). This may already be apparent in the non-breeding range of the continental Twite, which according to atlas data is receding to higher latitudes over time (Sonvon: Frater 2016; Vergeer and Marx 2018). A consequence of a shift to higher latitudes in the non-breeding range of continental Twite may be a widening of the divide in connectivity variance between the two groups subsequently preventing recruitment along the English Channel coast.

This study is the first to quantify migratory connectivity within the European Twite meta-population, and adding to the body of research on connectivity in the study of migratory and partial migrant species. Our study finds strong evidence for significant connectivity within the UK and, weak connectivity within the continental population. Disparities in migratory ecology are uncommon in the literature on connectivity, European Roller may be the only other documented within-species disparity; here, no migratory connectivity was found within the Spanish breeding group which freely mix on the non-breeding grounds in sub-Saharan Africa (Rodríguez-Ruiz et al. 2014), whereas on a continental scale, European Roller are found to have a strong migratory connectivity. This accounts for the distant breeding group located in the western Baltic region. Clearly, scale and sample size are influencing factors, with large scale studies more likely to find strong connectivity (Finch et al. 2017).

Within the Passerines, strong migratory connectivity is common (Macdonald et al. 2012; Stach et al. 2012; Hahn et al. 2013; Hallworth et al. 2015; Cormier et al. 2016), but weak (or low) migratory connectivity is also documented (Blackburn et al. 2017; Cooper et al. 2018). Of particular relevance to this study is the Snow bunting *Plectrophenax nivalis*, as they frequently form mixed flocks alongside Twite and Shorelark over the European non-breeding season (Dierschke 2002). North American Snow bunting were found have a strong migratory connectivity, forming an east west split during the non-breeding season (Macdonald et al. 2012), this is a pattern familiar to the British Twite and may relate to the shared winter site requirements. No research has been carried out on the migratory connectivity of the European Snow bunting population, however ringing records do support gene-flow over space and time between the American and British groups (Wernham et al. 2002). This model of strong connectivity is frequent in long distance migrants (Finch et al. 2017), however, weak migratory connectivity is also described in long-distance migrant passerines (Cooper et al. 2018) and may be an adaptation to environmental uncertainty (Blackburn et al. 2017). Despite the low sample size ($n = 29$), Whinchat *Saxicola rubetra* ringed in a single region of the non-breeding territory, 40km² of central Nigeria, went on to breed across an area of Europe equivalent to 26% of the continental landmass (Blackburn et al. 2017). Likewise, Kirtland's Warbler *Setophaga kirtlandii* ringed on two islands in the Bahamian Archipelago then intermixed heavily on the breeding grounds, regardless of winter island choice (Rockwell et al. 2012). Low migratory connectivity as an adapted response to environmental uncertainty would justify the connectivity model evident in the continental Twite proposed by this study (Atkinson 1998; J. Dierschke 2002; Dierschke and Bairlein 2004).

Condition and circumstance of recovery

The distribution of bird ring recoveries is heavily influenced by the landscape. Passive tags such as coded rings and colour marks require human observation to infer data from a marked individual. As a result, ring recoveries are often centred around human infrastructure and ringing stations, further confounded by the high percentage of ringed birds which are brought in by domestic cats (~42%) (Stenning 2018). The data from the EDB reflects this observation with 54% (77) of recorded predation events the responsibility of domestic cats. However, this still only represents 24% of recorded causes of mortality in Twite.

BTO Migration Atlas data was accessed in order to compare causes of mortality in the finches (Wernham et al. 2002). These data are interpreted differently, sorted in this case into four categories; domestic predator, deliberately taken by man, human related and other. Not only are these categories coarser than those presented in this study, but the data is 16 years older. However, these data do provide an insight into the mortality of related species, which is unlikely to change significantly over this time period. Of those species which associate with urban garden feeder throughout their annual cycle, Goldfinch, Bullfinch *Pyrrhula pyrrula* and Chaffinch, domestic predator and human related causes are most frequent in the data, however Goldfinch, is both a desirable caged bird and ubiquitous in urban areas and so is also frequently deliberately taken. The same applies to Linnet and to some degree Redpoll, which are both common domestic breeding stock but also, as Goldfinch, hunted in some regions where they are held as a delicacy (IUCN 2017). Twite in these data are also frequently deliberately taken and likely for the same reasons as the sister species. Human related causes of mortality also feature highly but among the eight species sampled, Twite have the highest percentage of mortality due to other causes which are not covered by the other three categories. Although the circumstances are not elaborated upon, Twite are the species which occupy the most extreme geographic and climatic niche of those sampled and are therefore perhaps more likely to fall prey to fluctuations within their environment.

Table 2.3. Cause of mortality for six Carduelinae and two Fringillinae finches. *Twite mortality data shown is reproduced from the BTO Migration Atlas and interpretation of data differs from that found in this study. Reproduced from the BTO Migration Atlas (Wernham et al. 2002).

Species	Domestic predator (%)	Deliberately taken (%)	Human related (%)	Other (%)
European Goldfinch	15	42	32	11
Eurasian Siskin	29	6	51	14
Common Linnet	13	44	32	11
*Twite	17	30	30	23
Common Redpoll	30	37	22	11
Common Bullfinch	30	22	39	9
Chaffinch	22	8	55	15
Brambling	11	30	43	16

Limitations to off-shore records

Because NSBC data is submitted by amateurs on an ad hoc basis from offshore infrastructure no effort can be made to verify the accuracy of either observers nor the observations submitted. Twite are a difficult bird to identify without prior experience and the apparent monomorphisms to the untrained eye between Twite and other species (Linnet, Reed Bunting *Emberiza schoeniclus* and Dunnock *Prunella modularis*) may prohibit accurate recording. Effort may also vary year on year depending on individual engagement with the scheme. Rapid technical expansion and automation in the offshore industry has resulted in fewer observers and therefore, the submission of records declined post 2008. Furthermore, fluctuations in oil prices and related redundancies have also affected data submission in recent years (A Thorpe, pers. comm.).

Abundant Scandinavian breeding passerines may also be under represented in off-shore data, it is likely that corpse displacement is more frequent in smaller species (Huppopp et al. 2016). Despite the unreliable nature of these data, known migrants (Common chaffinch, Brambling, European starling and Eurasian siskin) are highly visible in the records submitted, where other species such as Twite are not, this strongly implies that Twite are not regularly crossing the North Sea directly. Instead where mixing between the groups does occur it is more likely to be the result of stochastic movements either side of the narrow English Channel (see figures 2.7 & 2.8) Vagrancy is common in migrant and partial migrant species, and putative sightings from the Viking shipping region could easily be explained by Norwegian breeders being pushed offshore in adverse climatic conditions. Records from the south-east and north-west coast of England correspond to the non-breeding distribution of Twite within this region and are highly likely to be accurate. These data provide a valuable insight into the migratory ecology of European species, particularly those which utilise the Mediterranean and Eastern Atlantic flyways, a review of these data in their entirety and the quantification of reliability based on species present/absence data would be a highly valuable contribution to this field.

Limitations to EURING data

The amount of data to the EDB the constituent ringing schemes have contributed varies by region. This is limited by both the number of birds ringed and the number of subsequent recoveries. Where some schemes appear to have only submit records for birds that were then recovered elsewhere, others (particularly the BTO) have included recapture data.

Disparities in data is a clear limitation when submission is reliant on input from several sources, but general limitations to mark-recapture studies are synonymous across all of those sources and species. A general limitation to such studies, particularly regarding dispersal, is the imperfect detection across the species range. Where re-sighting data allows some degree of inference of distribution, this is unlikely to represent a random sample (Du Feu et al. 2016). There are circumstances in which recoveries can never provide a complete picture. In remote areas and those areas of low population, recapture and re-sighting effort can be greatly reduced and therefore bird species which use these areas can be under represented. These black spots are more frequent in eastern Europe, and Asia minor (Du Feu et al. 2016). As Twite distribution in Britain and continental Europe has a western bias, gaps in non-breeding recording are less likely. Where continental birds breed on the Scandinavian North Sea coast, access to breeding sites has prevented accurate recording of distribution and frequency relative to the non-breeding season.

Bird ringing or colour-marking with subsequent mark-recapture can provide highly valuable data for the continued study of avian demography, spatial and temporal ecology. The EDB therefore provides a mechanism for research at several scales, in this case population wide. However, limitations to data on this scale mean that the most value is derived when used in conjunction with other complimentary methods such as survey data, tracking or genetic studies.

Chapter 3

The Molecular ecology of Palearctic Twite

Introduction

The molecular ecology of the Finches has been heavily influenced by their biogeography, with the Pleistocene glacial period and subsequent Pleistocene/Holocene radiation particularly formative to present day diversity (Arnaiz-Villena et al. 2001; Zuccon et al. 2012). Improved accessibility to labs and a reduction in consumable costs have made molecular techniques an increasingly valuable tool to interdisciplinary conservation biologists; this is enhanced by advances in genomics and bioinformatics (Galla et al. 2016), and has allowed for greater clarity in the quality of intraspecific phylogenetic studies (Resano-Mayor et al. 2017; Tritsch et al. 2017; Aoki et al. 2018).

The use of DNA in the identification of, and relationships between birds has been well established since the early 1990's (Collinson 2017b). Genetic analysis has become particularly valuable in the identification of species limits in morphologically cryptic species, such as those belonging to the Reed *Acrocephalus* and Tree *Hippolais* warblers (Parkin et al. 2004) and the Nearctic flycatchers *Empidonax* (see Collinson 2017b). In the case of the former, marked differences in the mtDNA have allowed for the identification of the Caspian subspecies of reed warbler *Acrocephalus scirpaceus* as a vagrant in the Western Palearctic. MtDNA has also been invaluable in the exploration of diversity within polytypic species complexes, such as the Redpolls (see chapter 1, Marthinsen et al. 2008) and Lesser whitethroat *Sylvia curruca* (Olsson et al. 2013). Lesser whitethroat is an old-world warbler species with a continuous distribution across Eurasia, with at least five subspecies occupying varied climatic and habitat niches (Cramp 1992; Olsson et al.

2013). Olsen *et al.* (2013) identified robust genetic differences in the mtDNA cyt-b gene between all Lesser whitethroat subspecies, they found a 7% difference between the Eastern *S. c. blythi* and the nominate *curruca* and ~2.5 & between *blythi* and the central Asian *S. c. halimodendri*, concluding that these subspecies appear to have been genetically isolated for between one and four million years (Collinson 2017b; Olsson *et al.* 2013). Subsequent vagrant individuals captured during monitoring in Western Europe have been identified as belonging to three of five subspecies, suggesting high vagility if not gene-flow. Although some subspecies groups have a clear genetic signature, others have no detectable differences (Collinson 2017b). Likewise, examples exist of phenotypically divergent species showing almost no divergence in mtDNA (see, Irwin *et al.* 2009).

Current subspecies limits across the Palearctic

The IOC (Gill & Donsker, 2018) currently acknowledges nine subspecies (ssp.) of Twite, two European and seven across the Asian distributions. Although phenotypic differences exist between the European and Asian groups, here I address spatial differences between the currently considered subspecies. Within the European, *flavirostris* (Linnaeus, 1758) breeds along the north-west coast of Scandinavia as far north as extreme north-west Russia, the non-breeding season is spent along North Sea coast between southern Scandinavia and central France, occasionally extending into the central European interior (see figure 1.7). The British *pipilans* (Latham, 1787) has a locally fragmented breeding distribution, favouring moorlands and in northern parts, coastal habitats. During the non-breeding season the small breeding population in the Northwest of England move to the east and south-east coasts between the Humber estuary and Kentish coast. Twite breeding in north Wales, Ireland and the larger populations from the Hebridean islands and western Scotland, winter together along the west coasts of England and Scotland. A small number of birds breeding at the northern most tip of Scotland and the northern isles, once belonging to the now relict *bensonorum*, move down the coast of north-east Scotland. Within Europe morphological

differences are clinal and slight, *pipilans* is on average darker with more pronounced streaking in fresh plumage than the nominate *flavivrostris*.

The Asian groups are seldom studied and distributions less clearly defined. However, movements tend to be more nomadic and altitudinal in nature often forming sympatric flocks at lower altitudes (Cramp and Perrins 1994). Unlike the European group, Asian Twite primarily occupy an upland niche, associated with steppe, plateau and high altitude agricultural landscapes (Cramp and Perrins 1994; Orford 1973). *brevirostris* (Bonaparte, 1855) breeds in mountainous regions of northeast Turkey up to the Caucasus range in Georgia and down into the extreme north of Iran through Azerbaijan and Armenia. A small group also breed in the Turkish interior and western Syria, the non-breeding season is spent at lower altitudes (Beaman 1986; Porter and Aspinall 2010). *kirghizorum* (Sushkin, 1925) occupies northern and Central Kazakhstan, breeding in arid regions from the Volga-Ural Mountains eastwards and spends the non-breeding season in southern Kazakhstan and the north coast of the Caspian Sea (Wassink 2015; Gill and Donsker 2018). Although recognised as a full ssp., it is represented in the NHM collection as synonymous with *korejevi*. *korejevi* (Zarundny & Härms, 1914) breeds from Kazakhstan and Afghanistan to north-west China and winters at least in south-east Kazakhstan. The distribution of *korejevi* is largely uninvestigated (Wassink 2015; Gill and Donsker 2018) but altitudinal movements are highly likely. *altaica* (Sushkin, 1925) breeds between south-western Siberia and western Mongolia, seasonal movements are unknown but likely to be nomadic in nature (Gill and Donsker 2018). *montanella* (Hume, 1873) breeds between the Altai mountains, Kyrgyzstan and north-western China, extending into northern Pakistan and Afghanistan. *miniakensis* (Jacobi, 1923) breeds between western China and into eastern Tibet (Gill and Donsker 2018) and *rufostriata* (Walton, 1905) breeds between western and southern Tibet, extending into Northern India and Nepal (Gill and Donsker 2018), both are likely to undertake altitudinal movements as a response to climatic conditions.

Region variation occurs across the global range, but marked differences also exist between the European and Asian breeding populations. Where differences between the European birds are clinical, the 'lip-stick' pink rump of breeding males is synonymous throughout the region (Cramp and Perrins 1994). Within the Asian and Asia minor populations the breeding rump varies widely from a very pale pink to orange and even white. Ground-colour of these Asian birds is generally paler than those which breed in Europe, with more marked streaking (Cramp and Perrins 1994; Dunning, unpublished). Voice also differs between regions (Cramp and Perrins 1994; Marler and Mundinger 1975; Zablotskaya 1978), although lacking in contemporary data, the calls of European Twite were found to differ markedly to the calls of at least one Asian subspecies, *montanella* across several vocal components (Zablotskaya 1978).

Despite documented phenotypic variation and apparent allopatry, no genetic work has been carried out on this species prior to this study. However, studies of taxa which have similar phenotypic variation have found marked genetic differences between groups (Parkin et al. 2004; Olsson et al. 2013). Such studies have the potential to better inform conservation practice around isolated or cryptic species groups, see Collinson et al. (2017)

Methods

Sample collection

Primary samples were collected under license from wild caught Twite between 2015-2018; Secondary samples were collected by collaborators during the same period with the exception of the Swedish samples which were collected between 2005-2007. Three collection methods were employed in this study in order to obtain DNA samples, the collection of dropped feathers, plucking feathers and buccal swabbing. All British secondary samples and a number of primary samples were dropped feathers which ranged from small amounts of downy and/or contour plumage to primary flight feathers and rectrices, the latter being preferable. Swedish samples are made up of both plucked and dropped feathers, in some cases collected from dead birds. Local licensing in this region allows the extraction of two rectrices for genetic studies, and therefore the smallest samples from this region are larger than those collected in the British Isles. Within the British Isles, licensing only allows for the collection of up to four contour feathers from the rump. IsoHelix MS Mini DNA swabs (Isohelix, UK) were used on nestling and fledgling Twite, these swabs have small rectangular face (2mm x 6mm) which is specifically designed for high DNA yields from small organisms. Nestling birds readily accept swab insertion into the mouth, in adult birds a finger was used to gently hold the bill open whilst the swab was inserted. Where possible, the inside of the cheeks, under and around the tongue and the back of the throat were swabbed in a back and forth motion for 10-15 seconds, as was deemed appropriate for the birds size and to keep unnecessary stress to a minimum. Contour plumage was plucked with mid-way along the shaft using number one ringing pliers. These samples were then dry stored in individual, sterile Eppendorf tubes (Eppendorf, UK), or, in the case of some secondary samples (Machrihanish bird observatory) stored in 70-90% ethanol in individual glass specimen tubes or plastic specimen bags, all samples were then frozen at ~ -20. Swedish samples were dry stored in individual envelopes and then ordered by date and collection origin into larger padded envelopes before being frozen at ~-4.

During field work all samples were kept out of direct sunlight where possible and all samples were ultimately stored at -80 in dedicated freezers at the University of Nottingham, UK.

DNA extraction

Feather samples, buccal swabs and foot pad scrapings from museum samples were used to isolate DNA (for museum protocol see chapter 4). Extractions were carried out using the Qiagen DNA Micro kit (Qiagen, UK), with slightly amended protocols for extraction and sample digestion. Both the cleaning and the elution steps (following Qiagen protocols) used identical protocols between source sample types.

During feather growth in juvenile or moulting adult birds, the growing feather has a blood supply which is cut off at growth maxim. This blood vessel extends through the open inferior umbilicus (figure 3.1. D) and the hollow calamus (figure 3.1. C) where, on termination it leaves a small blood clot at the superior umbilicus (figure 3.1. B). Feather samples were defrosted and the calamus removed from the solid keratin of the rachis (figure 3.1. A), ~5mm above the superior umbilicus. The solid rachis and vane were then discarded as they do not contain any nucleated cells and the high surface area may hold contaminants. If the feather sample was plucked, the inferior umbilicus, which closes with the cessation of the blood supply is likely to have cells on the tip, however, as the avian erythrocyte is nucleated and therefore holds functional mitochondria (Stier et al. 2013), the former clotted region is likely to give increased DNA yields (Negro and Kalma 2005).



Figure 3.1 Feather anatomy as referred in this study. On the diagram A represents the solid central rachis and the lateral rows of barbs which make up vane. C is the hollow calamus, which begins at the proximal or inferior umbilicus (D) and terminates at a blood clot where the calamus meets the rachis known as the superior umbilicus (B).

The calamus was then cut with a sterile scalpel blade from the inferior to superior umbilicus (D to B) and then laterally into 3-4 smaller sections in order to expose these regions and internal calamus whilst retaining the proximal feather tip in adult samples. In smaller samples or where the aged feather calamus had become opaque the superior umbilicus was located using a microscope however it was often marked by a clear brown mark on the underside of the feather at the point where the barbs of the vane meet the calamus.

The prepared samples were then moved to a sterile Eppendorf tube. Where static charge caused small fragments to stick to the inside of the tube or lid, dry samples were pulse vortexed in a centrifuge for 10 seconds. 180µl of buffer ATL was then immediately added and left to equilibrate to room temperature (~20°C).

In order to effectively digest the feather keratin, 30µl of proteinase K, with the addition of an equivalent volume of dithiothreitol (DTT) to a 0.1 M concentration following Collinson *et. al* (2017, 2017a) and Shannon *et. al* (2014) was added to the solution in a fume cupboard. Samples were left in an incubation block, heated to 56°C and left to lyse for

at least 24 hours. Samples from larger birds or those from feathers with a longer and denser calamus were left for as long as 48 hours or until lysis was complete. Effective lysis causes the tissue to degrade entirely or in the case of more dense feathers become translucent and formless. Following cell lysis, 200µl of buffer AL was added to the lysate, it was then pulse vortexed for 20 seconds and returned to the heat block for 10 minutes at 72°C. The buffer AL and lysate must form a homogenous solution, where this didn't appear to be the case, samples were pulse vortexed for a further 20 seconds. 200µl of pure ethanol (96 - 100%) was then added and mixed in a pulse vortex for 10 - 20 seconds, this solution was then equilibrated to room temperature and left to incubate for five minutes. Following incubation, the solution was again pulsed in the centrifuge in order to dislodge condensation from the lid and tube sides.

In order to clean the lysate solution and the isolate DNA a series of washing steps were carried out following Quigen protocol with a Qiamp MiniElute spin column (Quigen, UK), these spin columns have a silica membrane which are designed to allow the elution and concentration of DNA from small volumes of source sample solution. Each lysate sample was transferred in its entirety to a sterile individual spin column being careful not to wet the rim and risk contamination. Any remaining soft keratin material from the lysing process was removed at this step. The lysate solution was then centrifuged at 8000 rpm (rotations per minute) for one minute. Each spin column is fitting into a single clean collection tube which is replaced and membrane flow-through discarded between spinning steps. Were the lysate has not passed through the membrane completely, the sample was centrifuged again at 14 000 prm for a minute, this was often caused by excessive material from the feather keratin left over from the lysing steps. Following the first spin step, 500µl of AW1 is added to the now dry spin column without wetting the rim, this sample was then replaced in the centrifuge at 8000 rpm for one minute. The second collection tube was replaced with a third clean one. The above step is then repeated following the addition of 500µl of AW2 buffer in the place of AW1. Contact between flow-through solution and the spin column membrane was avoided by careful handling and minimising vibration in newer centrifuge machines. After the third and final

spin at 8000rpm, the membrane is dried and ethanol carry-over removed from the eluate by spinning at 14 000rpm for a further three minutes without addition buffer added to the column.

The spin column was then removed from the collection tube and added to a sterile Eppendorf. All DNA samples were eluted in 30-80µl of Qiagen buffer AE and therefore buffer AE is equilibrated to room temperature to ensure the complete elution of bound DNA and added directly to the spin column membrane, before a further incubation for five minutes at room temperature. The spin column was then replaced in the centrifuge for at 14 000 for one minute. The additional incubation before the elution step generally increased DNA yield. Although secondary elutions were also prepared for some samples.

Dedicated pipettes of various capacities were always used for moving solution between vessels and tip replaced between all steps which involved DNA samples. During polymerase chain reaction (PCR) preparation a different group of dedicated pipettes were used and another group again for gel electrophoresis (see Polymerase Chain Reaction (PCR)). Likewise, contamination from outside of the lab was minimised by leaving lab coats inside the lab and regularly changing rubber gloves during sample and PCR preparation.

DNA was extracted from buccal swabs using the Qiagen Blood and Tissue extraction kit (Qiagen, UK), following a revised protocol by Kate Durant (unpublished). Although this method was broadly similar to that described above, some steps differ and spin columns are larger than those used for feather samples; prior to lysis, 20µl of proteinase K was added without additional DTT, swab tips were removed using dedicated pliers which were cleaned with 70% ethanol between uses and, following lysis are squeezed against the side of the tube in order to release absorbed liquid. The third spin step was at 13 000 for three minutes and the fourth drying step was not included. AE buffer was also maintained at 60°C throughout preparation and added warm to the spin column.

Following extraction, DNA concentration and purity was established using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Nucleic acids have a light absorbance maxima (λ_{max}) at around 260 nm. The ratio of this absorption at 280nm can therefore be used to measure the purity of both DNA and RNA, this metric is given as a 260/280 ratio, a score of ~ 1.8 is considered pure DNA and ~ 2 , pure RNA.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used to detect and amplify targeted segments of mtDNA prior to sequencing. 10 primer pairs were used during the course of this study, four of which are well established in avian phylogenetic research; Bird F1, Bird R1 (Herbert et al. 2004) and HC02198, LC01490 (Folmer et al. 1994) for the cytochrome c oxidase subunit I and OSCL1, OSCH2 (Herbert et al. 2004) for the cytochrome b regions of the mitochondrial DNA; P2, P8 are used widely in avian sexing (Griffiths et al. 1998). A fifth primer pair ATP5A1-F2, ATP5A1-R1 is seldom used but has recently been found effective in avian sex determination, specifically within the passerines (Bantock et al. 2008). Not all primer pairs successfully amplified DNA samples and so of these five only two; Bird F1, Bird R1, OSCL1 & OSCH2 were used in the sequencing of mtDNA. Primer pairs P2, P8 and ATP5A12-F2, ATP5A12-R1 were used to sex primary collected samples.

Table 3.1. Primer pairs used during sequencing of primary collected samples during the course of this study particularly Bird F1, Bird R1 and OSCL1, OSCH2.

Name	Gene/Region	Primer sequence 5'-3'
Bird F1	Cytochrome c oxidase subunit I	TTC TCC AAC CAC AAA GAC ATT GGC AC
Bird R1	Cytochrome c oxidase subunit I	ACG TGG GAG ATA ATT CCA AAT CCT G
HC02198	Cytochrome c oxidase subunit I	TAA ACT TCA GGG TGA CCA AAA AAT CA
LC01490	Cytochrome c oxidase subunit I	GGT CAA CAA ATC ATA AAG ATA TTG G
OSCL1	Cytochrome b	ATG GCC CTC AAT CTA CGT AAA
OSCH2	Cytochrome b	ATA GGA CTA GGA TGA TTG TGA AGT A
P2	Chromo-helicase-DNA-binding gene	TCT GCA TCG CTA AAT CCT TT
P8	Chromo-helicase-DNA-binding gene	CTC CCA AGG ATG AGR AAY TG
ATP5A12-F2	Avian ATP synthase α -subunit	GCT GTR GAC AGY TTG GT
ATP5A12-R1	Avian ATP synthase α -subunit	ATY GTT TYT GGT TRA TTA TTG

In addition to ordinary primers, see table 3.1, in order to sequence DNA from heavily degraded museum samples, a further five primer pairs were specifically designed to amplify a shorter region of the mtDNA (chapter 4).

Prior to PCR, all reagents were stored at 4°C unless otherwise stated by the manufacturer, in which case they were stored in a commercial freezer at -18°C. All PCR reactions were carried out in labelled filter tubes. 20µl PCR reaction contained, 2µl of combined 10x buffer + Mg Cl₂ solution (50mM), 2µl of additional Mg Cl₂, 2µl dNTPs, 0.5 forward and 0.5 reverse primers, 2µl of template DNA (dNTP) and 11.8µl of PCR quality H₂O (Sigma, UK) and 0.1µl of Taq polymerase enzyme. Taq polymerase reagents varied which resulted in minor amendments to protocol; taq gold was used for COI reactions which required a dedicated buffer, an additional 2µl Mg Cl₂ and a hot start to thermocycling, taq Roche for cyt-b reactions.

Thermal cycling for cyt-b (OSCL1 & OSCH1) and was programmed as follows; 3 minutes denaturation of DNA template at 95° C, followed by 40 cycles of a further denaturing step, 45s at 95° C, annealing of primers 45s at 55° C, and an extension of primers, 30s at 40° C. A final extension period was held at 70° C for 5m. However, a second program was also used for cyt-b as well as COI (BirdF1 & BirdR1) as follows; 2m initial denaturation at 95°C then 35 cycles of 35s at 95°C, 25s at 65°C, 1m 05s at 75°C followed by a final extension of 5m at 72° C, samples are then held at 4° C upon completion.

PCR products were electrophoresed on a 1% agarose gel (70mg agarose, 10x buffer and 2 µl of ethidium bromide) to visualise sample band sizes against a negative (H₂O) in order to detect any possible contamination of samples. Gel loading volumes varied by application, but were always with an equivalent volume of loading dye. A single well containing a DNA ladder of either 100bp or 1kb was used to measure band sizes.

Cleaning and sequencing steps

PCR products were then purified using Exonuclease I - Shrimp alkaline phosphatase clean-up prior to external sequencing. 1µl of SAP (Shrimp Alkaline Phosphatase), 0.15µl exoI (Exonuclease I) and 1.35µl SAP dilution buffer was added directly to the PCR products which showed up bands during gel electrophoresis. Exo I removes primers and SAP, remaining dNTPs leftover from the PCR process. Enzymes were stored at -18°C and immediately replaced following use. PCR products containing the ExoSAP mix were returned to the thermocycler where samples were incubated at 37°C for 30m followed by 95°C for 5m before storage at 4 °C. Following the ExoSAP purification step, samples are prepared for external sequencing with a BigDye v3.1 Terminator cycle sequencing kit. (Thermo Fisher scientific, UK), 2µl 5x buffer, 1µl BigDye, 0.5µl of primer, 3µl of purified PCR product and 3.5µl of PCR quality H₂O are added to new filter tubes to a volume of 10µl. These samples were then replaced in the thermocycler with an initial denaturation of 96°C for 1m, then 96°C for 10s, 50 °C for 5s and 60°C for 4m, cycled 24 times, a final hold of 4°C was also programmed to retain samples following completion. 10µl volume samples were then labeled and individually bagged for sequencing. Sanger sequencing was carried out externally by Edinburgh Genomics (University of Edinburgh).

Post sequencing

Sequences protein frequency distributions were visualised in BioEdit or Seaview.

Sequences which were close to the primer base pairs were manually edited according to peak clarity, where no peak was obvious, that region was removed from the individual sequence and during analysis, from the complete sequence alignment. Sequences were aligned using ClustalW and single nucleotide polymorphisms (SNPs) identified within the sequenced region.

Haplotype networks

Median joining haplotype networks were generated for both sequenced gene regions, cyt-b and COI as well as the control region (d-loop) for Twite, and COI for Marsh tit and Linnet using PopART haplotype network software (Leigh and Bryant 2015). Traits (in this case geographic origin data) were converted to .nex files and manually coded using DnaSP 6 (Rozas et al. 2017) and text edit software. Resulting networks show concatenated pie charts (nodes) each denoting a unique haplotype and linked via a linear network. The node size denotes the number of identical sequences within a given haplotype and the pie chart, the constitution of traits (in this case geographic distribution). A dash along a linear connection represents a single nucleotide polymorphism between haplotypes. Black nodes denote a median vector or ancestral haplotype as identified by the median joining network analysis, see Ferreri et al. (2011).

Genetic diversity & Tajima's D neutrality test

Haplotype (H_d) and nucleotide diversity (π) were carried out on Twite samples used during the course of this study for specific regions of the mitochondrial DNA and split by origin group, using DNAsp (Rozas et al. 2017). Nucleotide diversities and Tajima's D were also calculated separately using PopART (Leigh and Bryant 2015) for both Twite gene regions (cyt b & COI) sequenced as well as COI for to out-groups, the sister taxa, the Common Linnet and the Marsh tit. Nucleotide diversity (π) is the average proportion of nucleotide variation between randomly sampled sequence pairs. Tajima's D neutrality test represents the difference between the mean n -value of pairwise differences and the n -value of segregating sites. Tajima's D value population simulations predict that a group which is suffering a genetic bottleneck will show a higher D value (>0), where a group at a point prior to a bottlenecking event or in the period of subsequent recovery will produce a negative value (<0.01). This is caused by the removal of rare alleles selected for through a reduction in random sampling as a consequence of a bottleneck, or a greater reduction segregated sites than nucleotide diversity (leading to a positive D value). However, this means that a population with no selection pressure can appear similar to one which is recovering from a recent bottleneck (Tajima 1989).

Fixation index

Fixation index statistics (F_{st}) were estimated in Arlequin (Excoffier and Lischer 2010) and presented in this study with tables showing both pairwise differences and F_{st} bases haplotype frequency. F_{st} measures pairwise gene frequency in order to suggest the extent of gene flow which takes place between groups. Pairwise F_{st} can range from 0 to 1, where < 0 means complete mixing and infers subsequent gene-flow and 1 (a fixed population) suggests that there is genetic isolation between groups.

Samples

Additional samples were accessed from genebank for Twite (table 3.2), Common linnet (table 3.3) and Marsh tit (table 3.4). Haplotype sizes and in most cases collection location was accessed from published papers, Barcode of Life Data System (BOLD), museum collections or directly. Where precise location data or size was not available for each haplotype (particularly in the case of Qu, Lei & Zhang *et al.*), locations are given to the highest resolution available. In some cases, genetic analysis against known individuals suggested a region of origin (see genebank accession: FJ952420.1, FJ952409.1), for these samples, location is assumed and marked with an asterisk (*). Likewise, where exact sizes were unknown the size is marked with an 'n' on the tables which accompany the haplotype networks. Qu *et al.* (2010) sampled Twite from four locations; the three refugia edge regional groups of South Gansu, average altitude 3000m, East Qinghai region, average altitude 3400 m. and West Qinghai, average altitude 4200m, as well as an out-group, the Xinjiang region, average altitude 2000m.

Table 3.2. Secondary Twite sample haplotypes accessed from genebank. Where precise location is unavailable this is noted in the comments, sources are given as the surname of the lead author if published. Length shows the size of each segment as it was stored on Genebank.

Source	Gene	GB accession	length	location	comment
Price <i>et al.</i>	ND2	KJ455298	1041	Himalayan	Not used in this study
Price <i>et al.</i>	cyt b	KJ456172	1143	Himalayan	
Aliabadian <i>et al.</i>	COI	KF946624	693	Castricum, Holland	
Aliabadian <i>et al.</i>	COI	KF946625	693	Castricum, Holland	
Johnsen <i>et al.</i>	COI	GU571317	720	Hornoya, Norway	
Johnsen <i>et al.</i>	COI	GU571318	727	Ovre Heimdalen, Norway	
Johnsen <i>et al.</i>	COI	GU571794	648	Ottenby bird station, Sweden	
Qu <i>et al.</i>	cyt b	FJ952405	880	Qinghai-Tibetan plateau	Precise location unknown.

Qu et al.	cyt b	FJ952406	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952407	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952408	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952409	880	*Central Asia	Precise location unknown.
Qu et al.	cyt b	FJ952410	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952411	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952412	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952413	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952414	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952415	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	cyt b	FJ952416	880	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952417	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952418	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952419	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952420	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952421	652	*Central Asia	Precise location unknown.
Qu et al.	COI	FJ952422	652	*Central Asia	Precise location unknown.
Qu et al.	COI	FJ952423	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952424	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	COI	FJ952425	652	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	ctrl	FJ952426	288	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	ctrl	FJ952427	288	Qinghai-Tibetan plateau	Precise location unknown.
Qu et al.	ctrl	FJ952428	288	Qinghai-Tibetan plateau	Precise location unknown.

Qu <i>et al.</i>	ctrl	FJ952429	288	Qinghai-Tibetan plateau	Precise location unknown.
Qu <i>et al.</i>	ctrl	FJ952430	288	Qinghai-Tibetan plateau	Precise location unknown.
Kerr <i>et al.</i>	COI	GQ481476	679	Mongun-Taiginskiy Kozhunn, Tuva, Russia	
Kerr <i>et al.</i>	COI	GQ481477	680	Ovyurskiy Kozhuun, Tuva, Russia	
Kerr <i>et al.</i>	COI	GQ481478	692	Mongun-Taiginskiy Kozhunn, Tuva, Russia	
Kerr <i>et al.</i>	COI	GQ481479	694	Bai-Taiginskiy Kozhuun, Tuva, Russia.	
Liang <i>et al.</i>	COI	EU847714	1249	Daliugou, Jiashihexiang, China	
Drovetski <i>et al.</i>	ND2	FJ547506	1041	Tuva, Russia	
Mathinsen <i>et al.</i>	ctrl	EU400535	954	Heimdalen, Norway	
Mathinsen <i>et al.</i>	ctrl	EU400536	954	Heimdalen, Norway	
Mathinsen <i>et al.</i>	ctrl	EU400537	954	Hornoya, Norway	
Mathinsen <i>et al.</i>	ctrl	EU400538	954	Tromso, Norway	
Mathinsen <i>et al.</i>	ctrl	EU400539	954	Damshung, Tibet, China	
Mathinsen <i>et al.</i>	ctrl	EU400540	954	Damshung, Tibet, China	
Mathinsen <i>et al.</i>	ctrl	EU400541	954	Damshung, Tibet, China	
Mathinsen <i>et al.</i>	ctrl	EU400542	954	Damshung, Tibet, China	
Arnaiz-Villena <i>et al.</i>	cyt b	U83199	924	Caged bird, Antwerp, Belgium	possible introgression

Of Twite haplotype samples accessed, 45 were used in this study, exploring all available regions of the mtDNA all with the exception of ND2. Of 33 samples from Asia or Asiatic Russia, 26 were from the Qinghai-Tibetan plateau in east China and surrounding steppe (Qu, Lei & Zhang, 2016), more specific location details for these samples were unavailable. Nine Samples were from COI and 14 from cyt-b, all of which were aligned against primary samples. 13 haplotypes from the mitochondrial control region (d-loop) were aligned against each other for the first time.

Table 3.3. Linnet sample COI haplotypes accessed from genebank. Sources are given as the surname of the lead author if published. Length shows the size of the alignment used in subsequent analysis.

Source	Gene	GB accession	Length	Location	Comment
Dimitriou <i>et al.</i>	COI	KT803640	641	Cyprus	
Johnson <i>et al.</i>	COI	GU571787	641	Sweden	
Johnson <i>et al.</i>	COI	GU571308	641	Norway	
Kerr <i>et al.</i>	COI	GQ481454	641	Almaty oblysy, Kazakhstan	
Kerr <i>et al.</i>	COI	GQ481453	641	Kurskaya, Russia	
Kerr <i>et al.</i>	COI	GQ481452	641	Krasnodarskiy, Russia	
Kerr <i>et al.</i>	COI	GQ481451	641	Moscovskaya, Russia	
Johnson <i>et al.</i>	COI	GU571307	641	Norway	

Eight relevant Linnet samples were available on genebank, all of which relate to cytochrome oxidase region of the mitochondrial DNA. One of the haplotype samples was collected on the island of Cyprus, three others are from Scandinavia, one from European Russia and three from central Asia.

Table 3.4. Marsh tit sample COI haplotypes accessed from genebank. Where precise location data is unavailable this is note in the comments. Sources are given as the surname of the lead author if published. Length shows the size of the alignment used in subsequent analysis.

Source	Gene	GB accession	Length	Location	Comment
Johnsen <i>et al.</i> 2010	COI	GU571591.1	563	Akershus, Norway	
Johnsen <i>et al.</i> 2010	COI	GU572063.1	563	Bjotko-Arholma, Sweden	
Johnsen <i>et al.</i> 2010	COI	GU572062.1	563	Stockholm, Sweden	
Johnsen <i>et al.</i> 2010	COI	GU571590.1	563	Vest-Agder, Norway	
Kerr <i>et al.</i> 2009	COI	GQ482556.1	563	Kaliningradskaya, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482555.1	563	Kaliningradskaya, Russia	
Tritsch <i>et al.</i> 2016	COI	KX998526.1	563	Baden-Wuerttemberg, Germany	

Tritsch <i>et al.</i> 2016	COI	KX998525.1	563	Rheinland-Pfalz, Germany	
Tritsch <i>et al.</i> 2016	COI	KX998524.1	563	Rheinland-Pfalz, Germany	
Aliabadian <i>et al.</i> 2013	COI	KF946796.1	563	Europe	Precise location unknown.
Kerr <i>et al.</i> 2009	COI	GQ482557.1	563	Sakhalinskaya, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482551.1	563	Sakhalinskaya, Russia	
Paeckert <i>et al.</i> 2016	COI	KX687336.1	563	Muraviovka Park, Russia	
Tritsch <i>et al.</i> 2016	COI	KX998542.1	563	Beijing, China	
Tritsch <i>et al.</i> 2016	COI	KX998533.1	563	Primorskiy Kray, Russia	
Tritsch <i>et al.</i> 2016	COI	KX998514.1	563	Primorskiy Kray, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482553.1	563	Primorskiy Kray, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482552.1	563	Sakhalinskaya, Russia	
Paeckert <i>et al.</i> 2016	COI	KX687334.1	563	Muraviovka Park, Russia	
Saitoh <i>et al.</i> 2015	COI	AB843711.1	563	Japan	Precise location unknown.
Saitoh <i>et al.</i> 2015	COI	AB843100.1	563	Japan	Precise location unknown.
Saitoh <i>et al.</i> 2015	COI	AB843099.1	563	Japan	Precise location unknown.
Saitoh <i>et al.</i> 2015	COI	AB843098.1	563	Japan	Precise location unknown.
Tritsch <i>et al.</i> 2016	COI	KX998549.1	563	Tokyo, Japan	
Tritsch <i>et al.</i> 2016	COI	KX998541.1	563	Beijing, China	
Kerr <i>et al.</i> 2009	COI	GQ482550.1	563	Irkutskaya, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482549.1	563	Russia	Precise location unknown.
Tritsch <i>et al.</i> 2016	COI	KX998512.1	563	Near Kwangju, South Korea	
Kerr <i>et al.</i> 2009	COI	GQ482548.1	563	Primorskiy Kray, Russia	
Kerr <i>et al.</i> 2009	COI	GQ482547.1	563	Russia	Precise location unknown.
Paeckert <i>et al.</i> 2016	COI	KX687337.1	563	Muraviovka park, Russia	
Paeckert <i>et al.</i> 2016	COI	KX687335.1	563	Muraviovka Park, Russia	
Tritsch <i>et al.</i> 2016	COI	KX998513.1	563	Primorskiy Kray, Russia	

Saitoh <i>et al.</i> 2015	COI	AB843710.1	563	Japan	Precise location unknown.
Saitoh <i>et al.</i> 2015	COI	AB843709.1	563	Japan	Precise location unknown.
Tritsch <i>et al.</i> 2016	COI	KX998550.1	563	Tokyo, Japan	
Tritsch <i>et al.</i> 2016	COI	KX998534.1	563	Romerkastell Saalburg, Germany	
Aliabadian <i>et al.</i> 2013	COI	KF946797.1	563	Netherlands	Precise location unknown.

37 samples of Marsh tit were retrieved from the genbank repository. Nine samples relate to European Marsh tit, the remaining haplotypes are from Asian or Asian Russia. Several samples are missing precise origin data, these are therefore given at the highest resolution available.

Results

DNA extractions

During the course of this study, 106 DNA extractions were carried out on Twite feather samples and buccal swabs. Most samples (73%, $n = 78$) were below $10\text{ng}/\mu\text{l}$ in DNA concentration, leaving 26% ($n = 28$) above that level, 39% ($n = 42$) were below $5\text{ng}/\mu\text{l}$ of DNA. A sub-set of samples from Wales, United Kingdom, and all buccal swab samples failed to yield DNA concentration $> 2\text{ng}/\mu\text{l}$. Nanodrop results showing both DNA concentration ($\text{ng}/\mu\text{l}$) and 260/280 ratios for each sample for which an extraction was attempted. The lower dotted line indicates the approximate 260/280 ratio for pure DNA and the higher line shows the approximate ratio for RNA. The green line is a smoothed regression line between points with 95% confidence interval in the grey area. As DNA concentration increases to around $\sim 5\text{ng}$ the DNA purity as reflected by the 260/280 score stabilises, all samples with a 260/280 score which is outside of that expected by the model are confined to those of a DNA concentrated below $\sim 10\text{ng}/\mu\text{l}$, see figure 3.3.

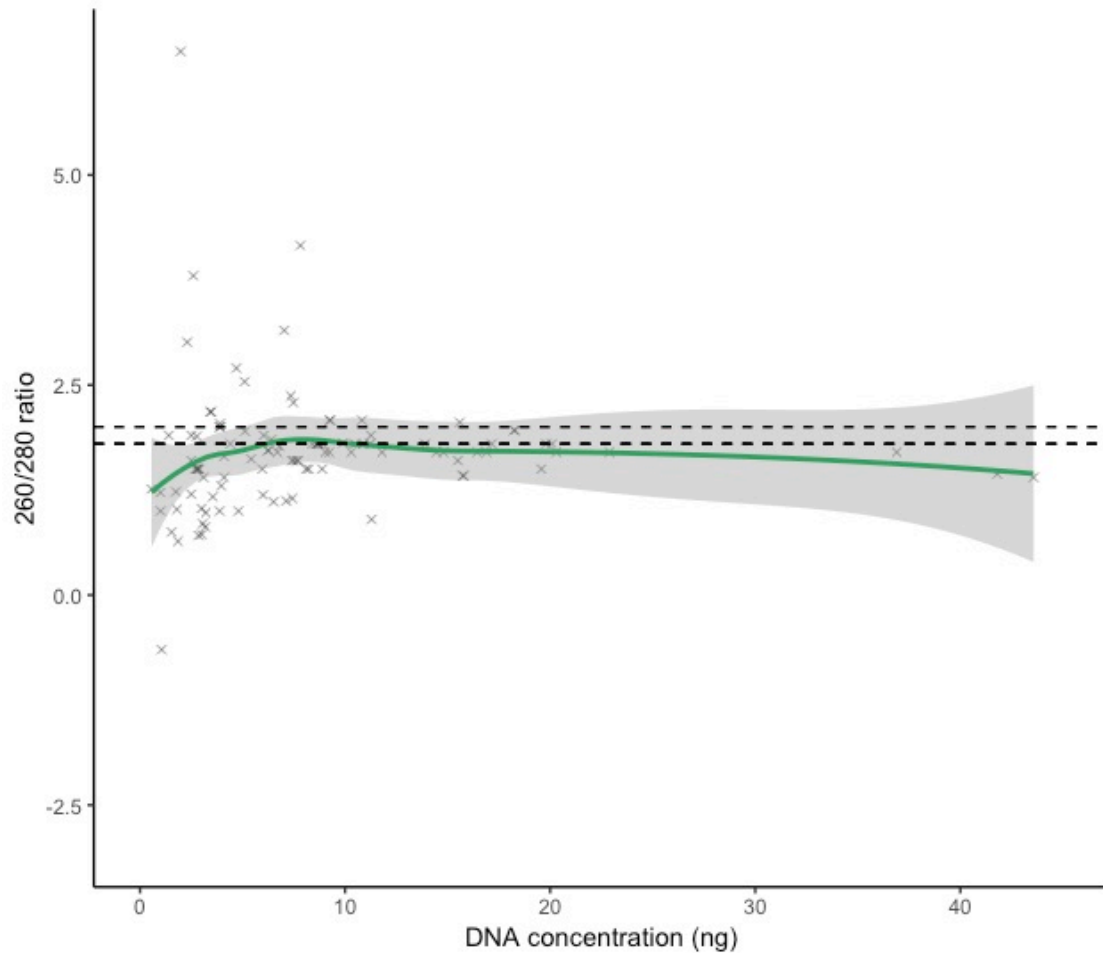


Figure 3.2. Nano drop scores showing the relationship between DNA concentration and 260/280 ratio. Where DNA concentration (ng/ μ l) is low 260/280 ratio is likely to suggest pure DNA (below ~ 1.8).

Haplotype diversity

Haplotype (H_d) and nucleotide diversity (π) was carried out on primary Twite samples used during the course of this study for specific regions of the mitochondrial DNA and grouped by origin as well as an out-group (Marsh tit). In both the Twite and the Marsh tit, both haplotype (H_d) and nucleotide diversity (π) are highest within the Asian groups when compared with the European. All groups had D values suggestive of a high frequency of rare alleles, recent population expansion or recent selective sweep ($D < 0$), however observed variation in the Asian Twite group is more similar to expected variation, suggesting little evidence of selection or recent population expansion.

Table 3.5. Table showing fragment sizes, nucleotide diversity (π), Theta per site (θ), the number of identified haplotypes, diversity within haplotypes, Tajima's D values and the number of samples within each group. For nine groups as defined by geographic origin. Note that the last two rows denote a comparative out-group, in this case Marsh tit.

Group	Gene	Fragment (bp)	Nucleotide diversity (π)	Theta per site (θ)	Haplotypes (n)	Haplotype diversity ($H_d \pm SD$)	Tajima's D	samples (n)
All Asian	Cytochrome oxidase subunit I.	0-624	0.01216	0.01188	12	0.987 (± 0.035)	0.103	13
All European	Cytochrome oxidase subunit I.	0-624	0.00602	0.01330	13	0.877 (± 0.074)	-2.16	19
British	Cytochrome oxidase subunit I.	0-624	0.00495	0.00766	8	0.891 (± 0.092)	-1.557	11
Continental European	Cytochrome oxidase subunit I.	0-624	0.00099	0.00141	4	0.714 (± 0.181)	-1.358	7
All Asian	Cytochrome b.	0-870	0.00430	0.00667	12	0.987 (± 0.035)	-1.504	13
All European	Cytochrome b.	0-870	0.00067	0.00130	5	0.511 (± 0.128)	-1.408	20
British	Cytochrome b.	0-870	0.00063	0.00108	4	0.495 (± 0.151)	-1.278	14
Out-group (Asian)	Cytochrome oxidase subunit I.	0-563	0.00435	0.00645	11	0.866 (± 0.042)	-1.110	27
Out-group (European)	Cytochrome oxidase subunit I.	0-563	0.00118	0.00235	3	0.318 (± 0.164)	-1.746	12

Haplotype networks

Median joining networks were generated to visualise relations between the haplotypes identified from both primary sequencing and those from previous studies listed on Genbank. The size of the nodes reflects the number of constituent individuals within a haplotype, with details for each found in the accompanying figure table.

A total of 624 bp nucleotides of the COI and 870 of the cyt-b were unambiguously resolved and used to identify 18 and 24 haplotypes respectively in three haplogroups. These haplogroups representing individuals from across the Twite's global range and reveal the genetic structure of this disjunct population for the first time. Note that central Asia, here includes Mongolia and parts of extreme northern China.

The cyt-b haplotype network clearly shows those that the 18 identified haplotypes form two markedly disjointed haplogroups; these between the European, represented by the pink and green nodes, and Asian populations, here represented by the teal nodes. A single haplotype is apparently closely related to the European haplogroup. This node represents a control population from the Xinjiang region of extreme NW China (central Asia), rather than the Qinghai-Tibetan plateau on the southern central border of which the remaining samples originate. The Asian group is more genetically variable than the European having 12 identified haplotypes against the European six. Within the European group there does not appear to be any unique genotype to British nor continental birds, despite the differing ecology and apparent isolation (see Chapter 2).

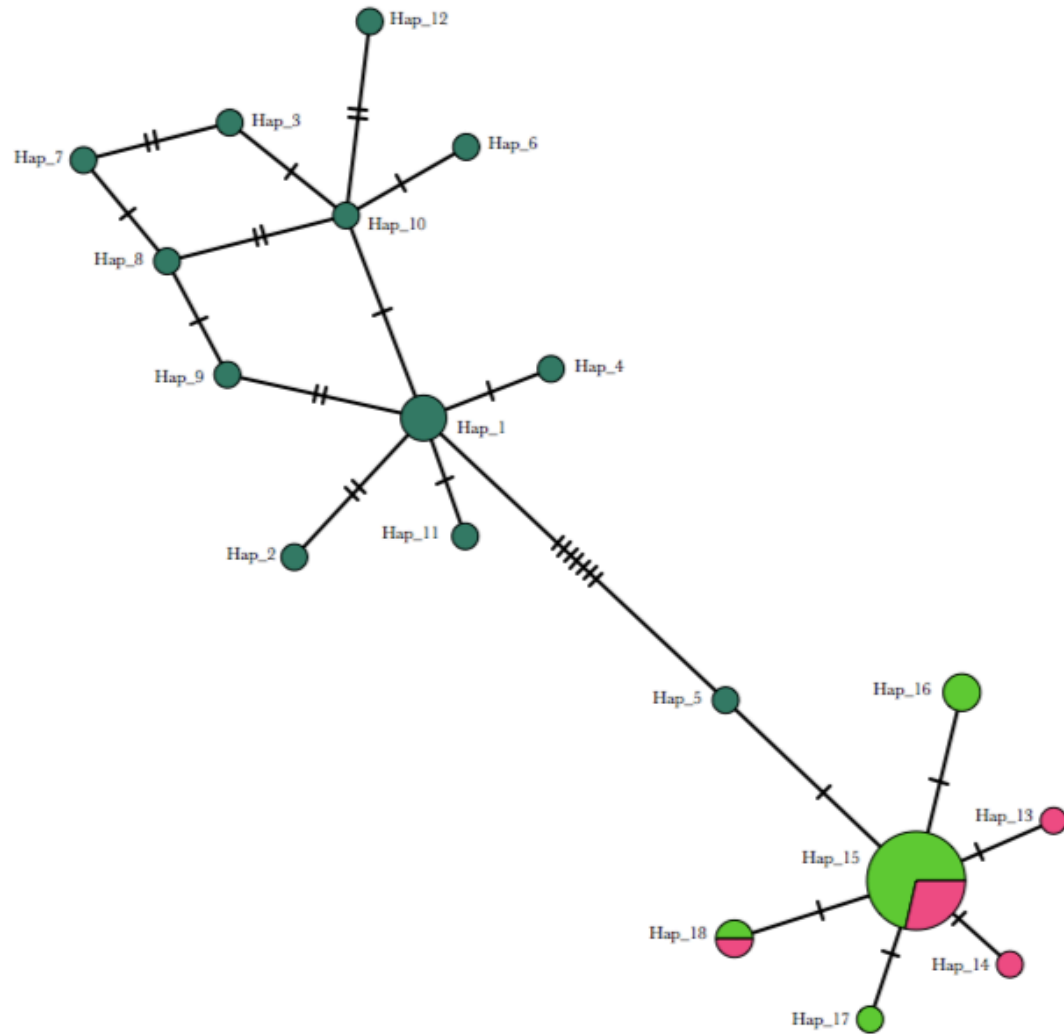


Figure 3.3. Concatenated haplotype network representing relationships between haplotypes identified within the cytochrome b region of the mitochondrial DNA of Twite. Sizes depict the number of individuals within the haplotype and colours the origin group of those individuals. In this case, pink represents continental European birds, pale green represents the British Twite and dark green are the assorted Qinghai-Tibetan plateau birds. Ticks on the connecting lines represent the number of bp differences between nodes. For details of haplotype constituents see table 3.6.

Table 3.6. Genbank accession numbers or primary sample codes and haplotype frequencies for haplotypes identified by this study within the COI region of the mtDNA for Twite. Note that n denotes that data on haplotype size was not made available.

Haplotype number	Haplotype frequency	Constituent sequences
1	2	KJ456172.1, FJ952405.1
2	n	FJ952406.1
3	n	FJ952407.1
4	n	FJ952408.1
5	n*	FJ952409.1
6	n	FJ952410.1
7	n	FJ952411.1
8	n	FJ952412.1
9	n	FJ952413.1
10	n	FJ952414.1
11	n	FJ952415.1
12	n	FJ952416.1
13	n	U83199.1
14	n	BU45934
15	14	CJ57103, CJ57105, L58399, L58640, L586335, Y316271, DH0, DH17, Oa652, Oa657, Oa689, 27110-05N, BY48151, RSPBa
16	2	DH22, DD30
17	1	Oa683
18	2	BY48083, DH42

The haplotype network generated for the COI region of the mtDNA identified 24 unique haplotypes. The form of this network is broadly similar to that of the cyt-b network (figure 3.5). Again, these haplotypes form two haplogroups which correspond to a European group comprising of red (British) and green (continental) nodes and an East Asian here represented by yellow nodes. However, a third haplogroup is apparent here, with the addition of data from the central Asian Russian/Kazakh steppe, here depicted in purple. This central Asian group forms four haplotypes around the out-group from the Tibetan study (Xinjiang region) of extreme north-west China and another from Shangyi county from northern China, near the inner Mongolian boarder (separated by ~2100km). This central Asian group is separated from the European cluster by only two bp differences, and the Tibetan plateau group by nine, despite relatively close geographic proximity. There are no discernible differences between the two European subspecies despite the increased number of haplotypes.

Both networks have large European haplotype with other, small nodes radiating off, this pattern is also suggestive of recent radiation, also suggested by the summary statistics carried out on these sequences, see, Results: Haplotype diversity.

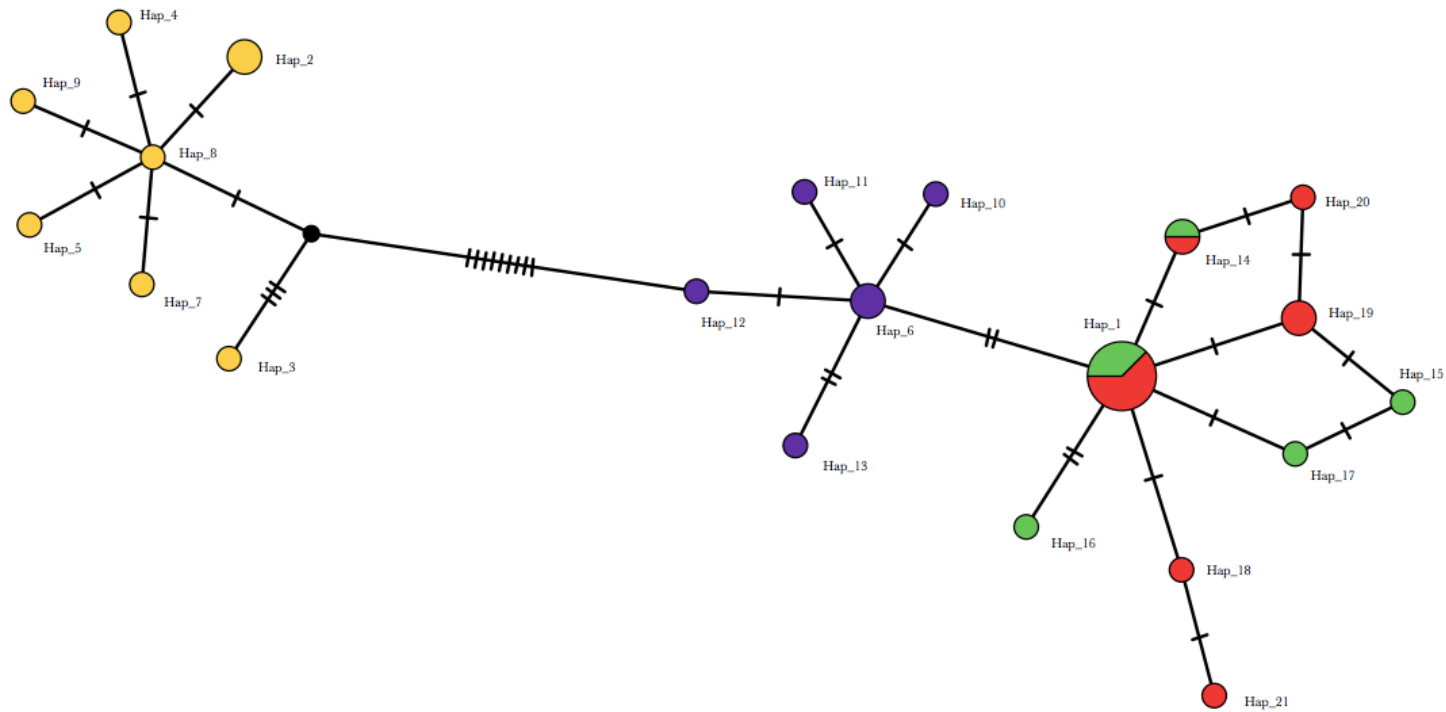


Figure 3.4. Concatenated haplotype network representing relationships between haplotypes identified within the cytochrome oxidase c subunit I region of the mitochondrial DNA of Twite. Sizes depict the number of individuals within the haplotype and colours the origin group of those individuals. In this case, yellow represents Asian birds from the Qinghai-Tibetan plateau, purple represents the Twite samples collected in eastern Russia, green are the continental European and red the British breeding birds. Ticks on the connecting lines represent the number of bp differences between nodes. For details of haplotype constituents see table 3.7.

Table 3.7. Genbank accession numbers or primary sample codes and haplotype frequencies for haplotypes identified by this study within the cyt-b region of the mtDNA for Twite. Note that n denotes that data on haplotype size was not made available.

Haplotype number	Haplotype frequency	Constituent sequences
1	n*	FJ952420.1
2	7	GU571794.1, GU571318.1, GU571317.1, L58635, Oa657, Oa683, Oa689
3	n	FJ952425.1, FJ952421.1
4	n	FJ952424.1
5	n	FJ952423.1
6	n	FJ952422.1, EU847714
7	n	FJ952419.1
8	n	FJ952418.1
9	n	FJ952417.1
10	1	GQ481479.1
11	1	GQ481478.1
12	1	GQ481477.1
13	1	GQ481476.1
14	1	CJ57103
15	1	RSPBa
16	1	BY48083
17	1	BY48151
18	1	CJ57105
19	1	DH0
20	1	DH17
21	1	DH22
22	1	L58399
23	1	Y316271
24	1	Z522590

Sequences made available on Genbank meant that a haplotype network for the mtDNA control region (d-loop) could be generated. Two haplogroups apparent from haplotypes, an Asian which here is split between haplotypes two and three and a European represented by the remaining haplotype. The difference between the two groups is only 4bp, the Asian samples in this case are all from Damshung, Tibetan region of China (and not the Qinghai Tibetan plateau) and the European only represented here by Norwegian birds. It is unclear if these Asian birds are representative of those clearly divergent haplotypes from the Qinghai Tibetan plateau (see figures 3.5 and 3.6) or more closely related to central Asian.

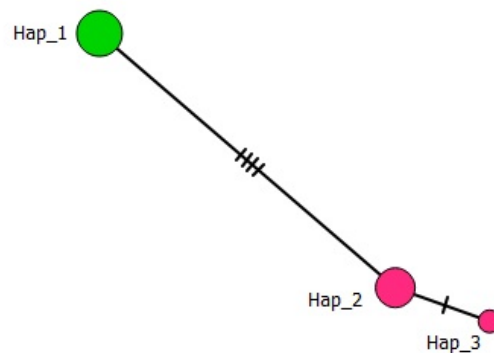


Figure 3.5. Concatenated haplotype network representing relationships between haplotypes identified within the mtDNA control region (d-loop) of Twite. Sizes depict the number of individuals within the haplotype and colours the origin group of those individuals. In this case, green is the continental European Twite and pink the Asian group(s). Ticks on the connecting lines represent the number of bp differences between nodes. For details of haplotype constituents see table 3.8.

Table 3.8. Genbank accession numbers haplotype frequencies for haplotypes identified by this study within the cyt-b region of the mtDNA for Twite.

Haplotype number	Haplotype frequency	Constituent sequences.
1	4	EU400535, EU400536, EU400538, EU400537
2	3	EU400539, EU400540, Eu00541
3	1	EU400541

The Marsh tit is a passerine with a similarly disjunct population to that of the Twite. Haplotype data was accessed from several projects which made data available on Genbank, although only comparable haplotypes exist for the COI region of the mtDNA. Within the cytochrome oxidase region of the mtDNA, the European and Asian breeding group are much more closely related than the equivalent groups in Twite, which clearly form distinct haplogroups. The European samples in green are separated from the Asian/ Asiatic Russian samples in red and purple respectively by only 2 bp differences. Although there does appear to be a distinct European haplogroup, the Asian and Russian samples are closely related. Although sampling regions are not equivalent, the most divergent Twite group, from the Qinghai Tibetan plateau, were sampled from a region where Marsh tit are absent. The majority (10) of the European Marsh tit form a single haplotype made up birds from Scandinavia and the European interior.

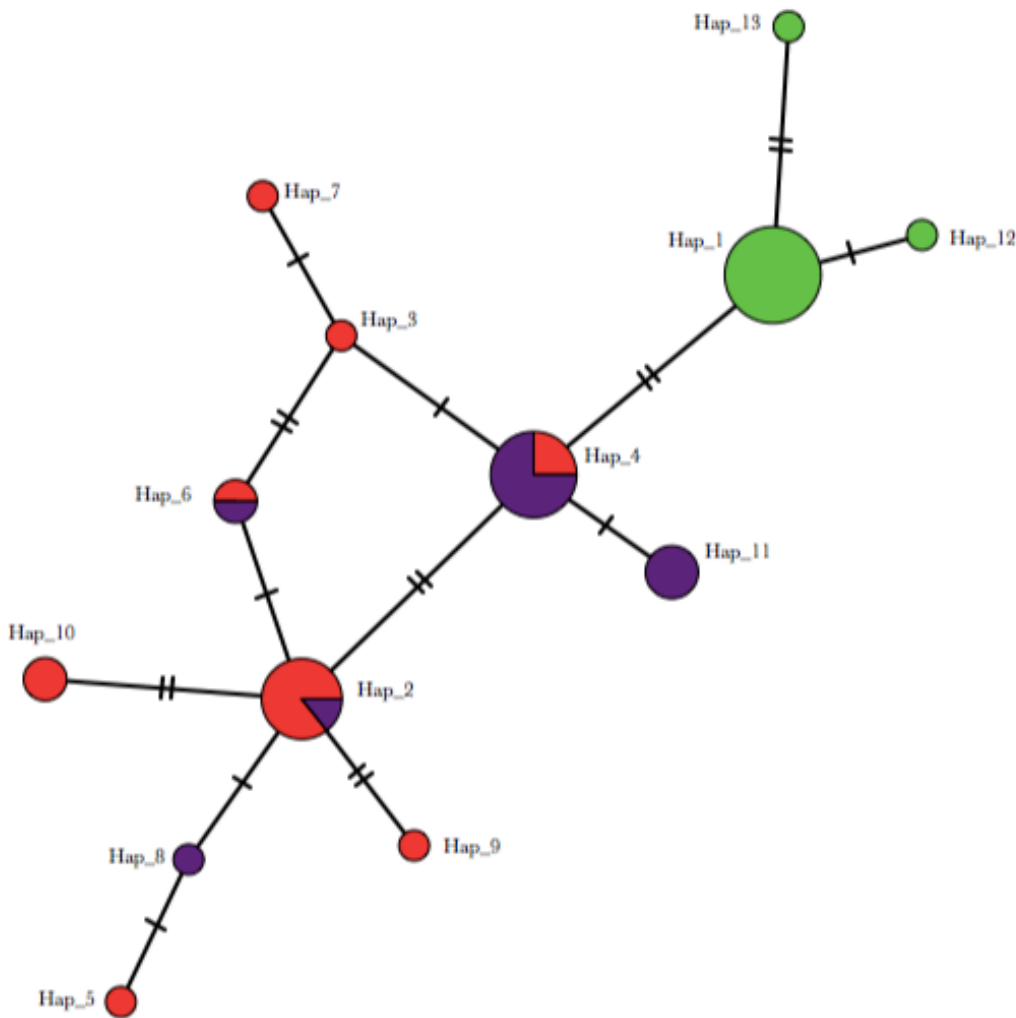


Figure 3.6. Concatenated haplotype network representing relationships between haplotypes identified within the COI region of the mitochondrial DNA of Marsh tit. Sizes depict the number of individuals within the haplotype and colours the origin group of those individuals. The European Marsh tit form a distinct haplogroup (green), the Asian (red) and Russian (Purple) are closely related but have less structure. Ticks on the connecting lines represent the number of bp differences between nodes. For details of haplotype constituents see table 3.8.

Table 3.9. Genbank accession numbers haplotype frequencies for haplotypes identified by this study within the COI region of the mtDNA for Marsh tit.

Haplotype number	Haplotype frequency	Constituent sequences
1	10	GU571591.1, GU572063.1, GU572062.1, GU571590.1, GQ482556.1, GQ482555.1, KX998526.1, KX998525.1, KX998524.1, KF946796.1
2	7	GQ482557.1, GQ482554.1, GQ482551.1, KX687336.1, KX998542.1, KX998533.1, KX998514.1
3	1	GQ482553.1
4	8	GQ482552.1, KX687334.1, AB843711.1, AB843100.1, AB843099.1, AB843098.1, KX998549.1, KX998541.1
5	1	GQ482550.1
6	2	GQ482549.1, KX998512.1
7	1	GQ482548.1
8	1	GQ482547.1
9	1	KX687337.1
10	2	KX687335.1, KX998513.1
11	3	AB843710.1, AB843709.1, KX998550.1
12	1	KX998534.1
13	1	KF946797.1

A Haplotype network was also generated for what little haplotype data was available for the Common linnet. Despite having a continuous distribution across Eurasia, they are the closest interspecific taxonomic affiliation to the Twite. Here the European samples in green share haplotypes with their Asian (Kazakh and Russian, in red) conspecifics. A single haplotype represents the subspecies *autochthona* in this case from the island of Cyprus.

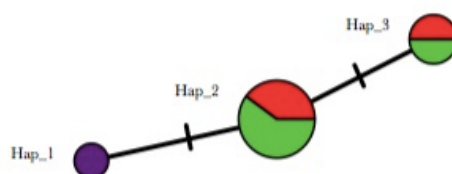


Figure 3.7. Haplotype network representing relationships between haplotypes identified within the cytochrome oxidase region of the mitochondrial DNA of Common linnet. Sizes depict the number of individuals within the haplotype and colours the origin group of those individuals. In this case, purple represents a ssp. from Cyprus, green represents the European samples and red, eastern Russia and Kazakhstan. Ticks on the connecting lines represent the number of bp differences between nodes. For details of haplotype constituents see table 3.10.

Table 3.10. Genebank accession numbers haplotype frequencies for haplotypes identified by this study within the COI region of the mtDNA for Common Linnet.

Haplotype number	Haplotype frequency	Constituent sequences
1	1	KT803640
2	5	GU571787, GU571308, GQ481454, GQ481453, GQ481452
3	2	GU571307, GQ481451

The distribution of SNPs within the sequenced mtDNA

Following alignment, a number of single nucleotide polymorphisms (SNPs) were identified within the sequenced regions of the genome, the distribution of which is mapped (see figures 3.8a. & 3.8b.). Although a number of these SNPs are individual variation or sequencing errors and are therefore unrelated to biogeography, others; COI, positions 144, 183, 228, 332, 364, 428, 506 & 577 and cyt-b, 257, 488, 581, 653, 831, 883, 915, 990 & 1017 are all strongly suggestive of either an Asian or European origin. However, the central Asian samples haplotypes (see figure 3.4) are exceptional in that they do not follow this rule. Despite close geographic proximity to the Tibetan haplogroup they are more closely related to the European. Throughout the cytochrome-b region of the mtDNA a large region between position ~170 and ~270 is largely conservative, smaller regions between ~270 and ~600 are also mostly conserved (figure 3.8a). SNPs are more frequent in the third codon position however, after position ~625 the genetic variation is more marked in the first and second positions. The frequency of SNPs in the COI region is greater and more varied in codon position, although as with the cyt-b the third codon position is more frequently the location of SNPs.

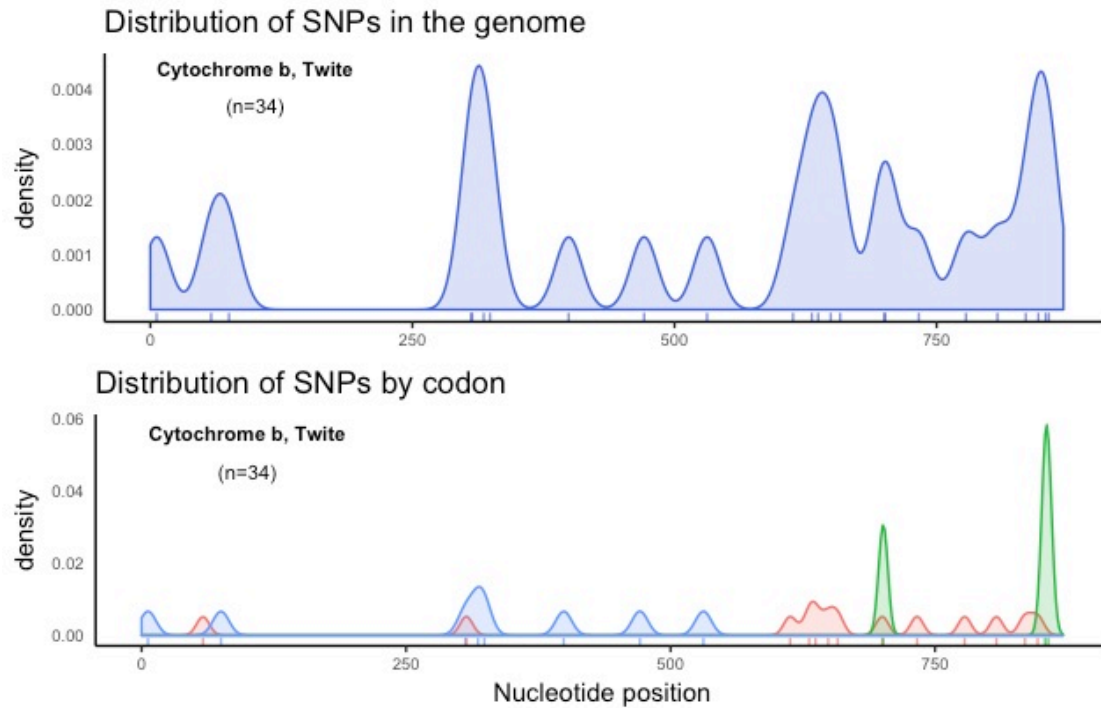


Figure 3.8a. Distribution of SNPs in the sequenced cytochrome b region of the mitochondrial DNA, the top pane shows SNP distribution within the sequenced region and then by codon position codon where red is the first position, green the second and blue the third.

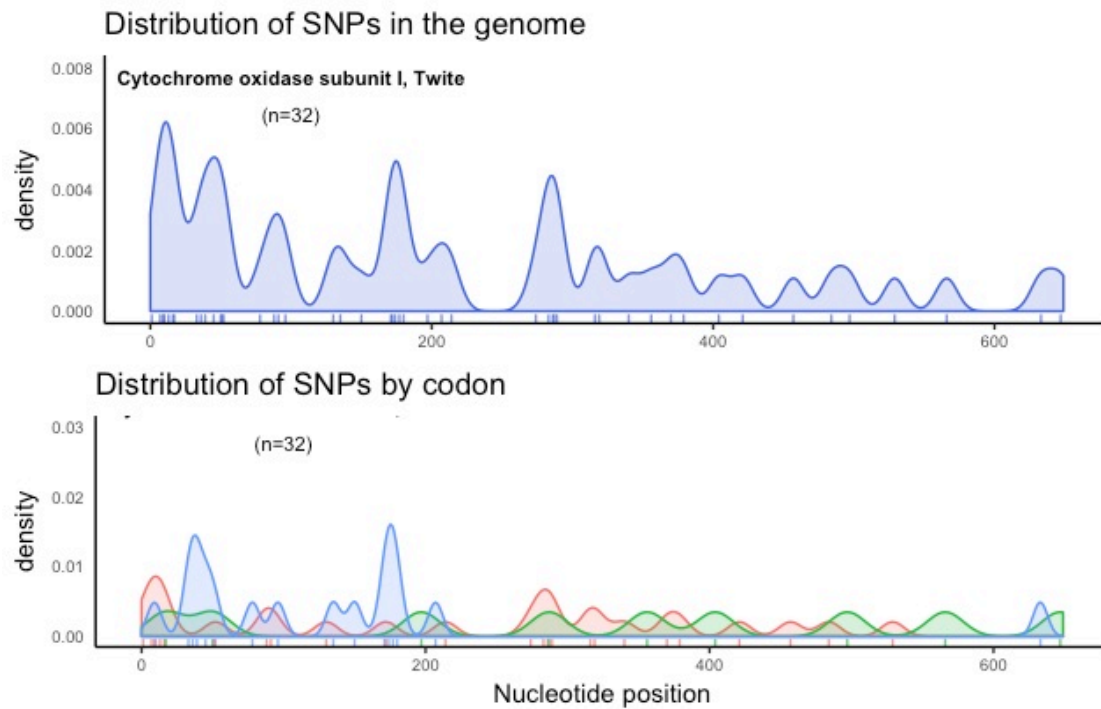


Figure 3.8b. Distribution of SNPs in the sequenced COI region of the mitochondrial DNA, the top pane shows SNP distribution within the sequenced region and then by codon position codon where red is the first position, green the second and blue the third.

Fixation index

Fixation index metrics (F_{st}) were estimated for the COI region of the mtDNA in Marsh tit and COI and cyt-b regions in Twite, first population pairwise (pw) F_{st} and then from haplotype frequencies (hf). The greatest geographic distance between two groups of Marsh tit was between the Scandinavian and East Asian groups and is reflected in the F_{st} index (pw 0.66/ hf 0.53). Haplotype frequencies were less accurate within Twite groups due to low sample sizes, but pairwise F_{st} remained a robust indicator of population structure. The same distance in the Twite group, here between continental Europe (though all Scandinavian breeders) and east Asia (Tibet) found greater genetic distance between groups (COI pw: 0.73 & cyt-b pw: 0.71). The genetic distance between the central Asian/Russian groups and the neighbouring East Asian groups was also greater between Twite groups (COI pw: 0.24) than Marsh tit (pw: 0.16). Both Twite and Marsh tit were found to form a panmictic population across Britain and continental Europe (Twite) and between Scandinavia and continental Europe (Marsh tit) ($F = < 0$).

Table 3.11. Genetic structure (F_{st}) of sequence pairs for COI dataset among four Marsh tit populations. Numbers above the diagonal matrix are conventional F_{st} from haplotype frequencies and those below are population pairwise F_{st} .

Marsh tit (COI)	Scandinavia	Continental Europe	Russia	East Asia
Scandinavia	-	0.01	0.3	0.53
Continental Europe	0.0	-	0.15	0.35
Russia	0.46	0.46	-	0.12
East Asia	0.66	0.63	0.16	-

Table 3.12. Genetic structure (Fst) of sequence pairs for COI dataset among four Twite populations. Numbers above the diagonal matrix are conventional Fst from haplotype frequencies and those below are population pairwise Fst.

Twite (COI)	British	Continental Europe	Central Asia	East Asia (Tibet)
British	-	0.0	0.11	0.08
Continental Europe	0.0	-	0.1	0.07
Central Asia	0.41	0.4	-	0.03
East Asia (Tibet)	0.71	0.73	0.24	-

Table 3.13. Genetic structure (Fst) of sequence pairs for cyt-b dataset among four Twite populations. Numbers above the diagonal matrix are conventional Fst from haplotype frequencies and those below are population pairwise Fst. Note that a single central Asian specimen is included with the Asian samples here, which are otherwise all from East Asia

Twite (cyt-b)	British	Scandinavia	Asian (Inc. Russia)
British	-	0.0	0.23
Scandinavia	0.0	-	0.17
Asian (Inc. Russia)	0.77	0.71	-

Discussion

Marthinsen et al. (2008) suggested that the two Twite subspecies used as an out-group in their study on Redpoll taxonomy constituted two well-supported monophyletic groups. Despite this chance observation, the genetic structure of the global Twite population has remained unresolved. This study finds that across the Eurasian mid-latitudes, Twite form at least three haplogroups based on five of nine current subspecies. Although within Europe and central Asia these subspecies are not distinguishable based on mtDNA alone, the population on the Qinghai Tibetan plateau are highly divergent. This study finds that the extent to which Tibetan Twite differ from their conspecifics is not paralleled in the Marsh tit, which has a similar distribution, nor the Common linnet, which has a continuous distribution across Eurasia. Furthermore, central Asian Twite are more closely related to the European haplogroup, some ~5700km to the east than the those found on the Qinghai Tibetan plateau, ~1050km to the south, despite geographic proximity. Sub-specific limits and distributions are poorly understood and often synonymised in Twite, particularly within the Asian group. This study was unable to include any samples from Asia minor but these populations are expected to be genetically similar to the European and central Asian birds.

Instability in Tajima's D value statistics and the arrangement of haplotype networks also suggests recent rapid radiation in both the central Asian and European groups. This corroborates Qu et al. (2010), who reported a geographic break between Twite populations corresponding to the Asian and central Asian haplogroups in this study; the distribution of Twite on the Qinghai-Tibetan plateau may not have been affected by Pleistocene glacial cycles, but they may have prevented gene-flow between the two Asian haplogroups. This would explain the rapid radiation from Asia across Western Europe and may also suggest a Tibetan origin for the central Asian and European Twite.

Comparison with Marsh tit

Genetic samples were compared from the mtDNA of Twite and Marsh tit, both of which have a wide but disjunct distribution across Eurasia. Evidence of current gene-flow (or gene-flow which existed until very recently) was apparent between British and continental European Twite. Central Asian populations differed from the European in both species but more markedly in the Twite. Because of discrepancies in distribution between species, geographic distance is not a reliable comparative metric, however, it is clear that gene flow between Twite groups globally is limited, where, for the Marsh tit, this gradient is more continuous over geographic distance. It is also apparent that the Tibetan population of Twite is highly divergent from the European and central Asian birds, Marsh tit is absenting from this region so could not be directly compared.

Comparison with other species complexes

Phylogenetic relationships within the Redpoll species complex are well studied (Seutin et al. 1995; Ottvall et al. 2002; Marthinsen et al. 2008). Unlike the single species taxonomic groups addressed in this study, the Redpoll were once considered to form three distinct species based on evidence for assortative mating (Lifjeld and Bjerke 1996) and secondary contact following expansion from Scandinavia (Knox et al. 2001). However, despite these findings, mtDNA fails to support full species status between two of the most common subspecies, the Common/Mealy and Lesser redpolls (Seutin et al. 1995; Ottvall et al. 2002; Marthinsen et al. 2008). What genetic diversity was found was likely the result of sudden post-glacial population expansion during the Pleistocene epoch (Ottvall et al. 2002). The most recent study on Redpoll taxonomy found no genetic structure in either haplotype network analysis nor individual-based analysis of microsatellites see, figure 3.9 (Marthinsen, Wennerberg, and Lifjeld 2008).

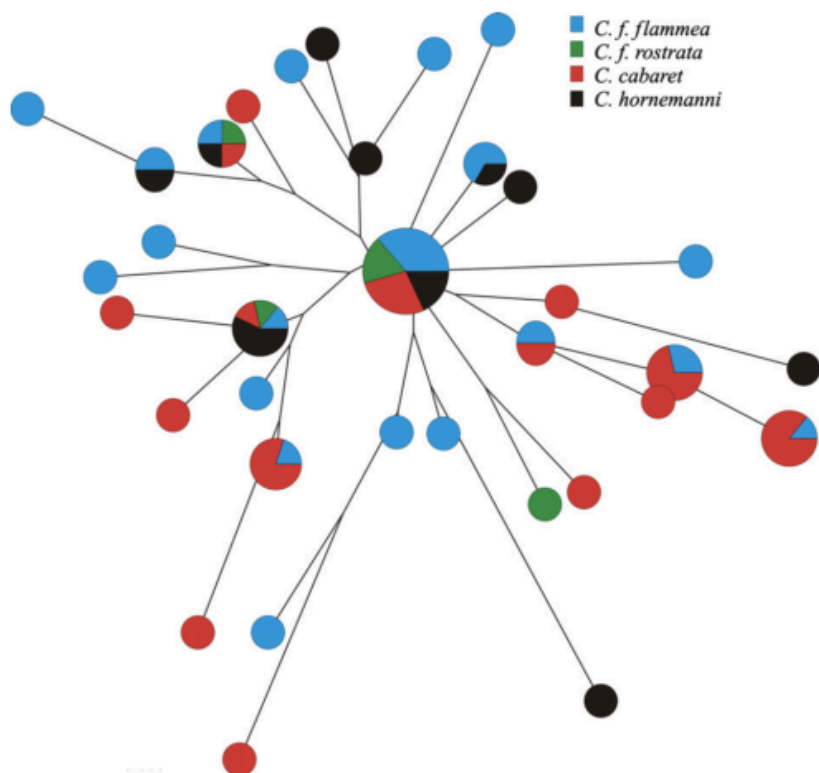


Figure 3.9 Neighbour joining tree for mtDNA control region (d-loop) haplotypes from four redpoll taxa. Reproduced from Marthinsen, Wennerberg & Lifjeld (2008), for frequencies see source; Appendix A.

All redpoll clades identified by Marthinsen et al. (2008) had low bootstrap support (< 64%), and all haplotype clusters had representation from at least two if not all Redpoll taxa. The study also failed to find any genetic divergence within the redpoll species complex in either mtDNA or microsatellites.

Another well studied species complex is that of the Lesser whitethroat. Much like Redpoll and the Twite the Lesser whitethroat is a polytypic species which occupies much of the northern, central and Eastern Palearctic region (Cramp 1992). Of five currently recognised subspecies, Olsson et al. (2013) found that all were identifiable by markers in the cyt-b region of the mtDNA. Of the Twite sampled by this study, informative bp differences were identified between groups in western Europe, central Asia and the Tibetan plateau, but only the latter corresponded to a single subspecies.

Qu et al. (2010) reported mixing of Twite sampled from three regions of the Qinghai-Tibetan plateau, however whether these haplotypes constitute a single subspecies has not been made available by the study. It is expected that further sampling will reveal that the Twite found in Asia minor will form a group with those of central Asian origin presented here, although this remains speculative

Molecular limits in Eurasian bio-geography

It has been suggested that Twite are the only European bird derived from the Tibetan fauna type (Sharrock 1976; Holland, Spence, and Sutton 1984) and the genetic distance and Tajima's D value presented by this study may corroborate the theory (see results: Haplotype diversity & Discussion), but how this biogeography fits within Pleistocene vicariance is unclear. Studies of isolated species groups are less informative in the terms of global biogeography (Qu et al. 2010), a more relevant approach is to address widespread populations or species complexes (Song et al. 2018). Recent studies on the *Pica* Magpie complex, a cosmopolitan group of Corvids *Corvidea*, have found four major haplogroup lineages based on mtDNA, the European-Siberian, south-east Asian, north Africa and North America (Kryukov et al. 2017). Although sample sizes from north America and China were low ($n = 1$). Song et al. (2018) continued this research using two mtDNA markers, with more comprehensive sampling across Eurasia and North America. This study found that the North American clade of *Pica* Magpies is sister to that of the northern Eurasia, while populations in Arabian and Qinghai-Tibetan form unique sister clades (Song et al. 2018). These ancient isolated groups of magpie in the Arabian Peninsula and the Qinghai-Tibetan plateau, diverged from the primary clade during the late Pliocene to the middle Pleistocene glacial period, which was also formative in the biogeography of Twite (Newton 1972; Cramp and Perrins 1994). Gene flow between Magpie groups was inferred and mapped based on mtDNA diversity see, figure 3.9. This analysis found that the Qinghai-Tibetan plateau, represented in figure 3.9: node blue 11, holds an isolated group of Eurasian magpie *Pica pica*. By way of comparison the region where central Asian Twite were sampled in Qu et al. (2010) is nearest to node orange 19. Qu et al

(2010) found significant isolation by distance within Twite on the Tibetan plateau ($r = 0.986$, $p = 0.012$) which was not detected when the central Asian out-group was removed from the analysis ($r = -0.2469$, $p = 0.814$). Furthermore, this out-group constituted a genetic outlier when data was visualised with maximum parsimony networks. The bio-geographic finding within the magpie complex (figure 3.9) broadly correspond to those briefly suggested as divergent in Twite groups by Qu et al. (2010) and corroborated by this study.



Figure 3.10. Map showing gene flow among geographical populations of Eurasian magpie *Pica pica* in north Eurasia (orange) and east Asia (blue). Codes consistent with population indices for additional analyses within the study. Arrows indicate directions of asymmetrical gene flow. May suggest a similar mechanism for Twite which seem to show a divergent group in east Asia. Reproduced from Song et al. (2018).

Chapter 4

DNA from museum specimens

Introduction

Museum collections provide a valuable resource of avian genetic material in the non-destructive tissue extraction from the feet of museum bird specimens (Munday et al. 1997). In addition to the sequencing and alignment of modern samples (Chapter 3), ancient DNA was extracted from 25 Twite specimens originating from the Natural History Museum (NHM) in Tring, United Kingdom. These samples were selected to provide historical context to contemporary analysis as well as filling in gaps in the current distribution of samples, most notably the Asia minor group comprised of *brevirostris* & *korejevi*.

Methods

Sampling and DNA extractions

Extractions were carried out using the Qiagen DNA Micro kit (Qiagen, UK), but followed an amended protocol to that set out in chapter 3 for primary DNA samples. Museum specimens were requested to represent the entire global distribution of Twite based on what subspecies were available within the NHM collection at Tring (figure 4.1). At least three specimens from each major region/subspecies were selected based on appearance, age and quality of labelled information, over two visits to the NHM in July 2017 and March 2018. Skin specimens are stored dry in ordered drawers which are in turn held in temperature controlled cabinets. Contamination of DNA is kept to a minimum by keeping species separate but collections may have been mixed historically during periods of movement or prolonged storage.

Sampling was carried out by collaborators at the NHM Tring using sterile blades in a clean environment. Each 2-4mm sample was cut from the toe pad on the underside of the foot from the selected Twite specimens. Samples were then stored in individually marked specimen tubes before being sent to the university for DNA extraction and analysis. Samples were cut in half where possible, again using a new sterile blade for each and in an environment which was cleaned with 70% ethanol between sample handling.

Samples were then cut into smaller pieces and incubated in 100µl of distilled PCR quality water for one hour at 57°C prior to the lysing stage. This allowed the tissue to soften and aid digestion downstream. These tissue samples were lysed in protease K without the additional DTT, otherwise extraction and post extraction steps remained the same as those described in chapter 3. DNA concentrations and 260/280 ratios were subsequently measured on a Nanodrop (Thermo Fisher Scientific, UK).

Table 4.1 Samples taken from the toe-pad of the NHM Twite samples, NHM accession numbers and subspecies according to the specimen labels. Ages and sequenced positives are also shown here, all those sequenced were with Twite Fo5, Twite Re5 primer pairing and marked with a 'Y' if successfully applied. Ages are unclear so ordered based on accession into the museum collection.

Specimen	NHM accession	Type	Amplified	~Age
Carduelis flavirostris (Linnaeus, 1758)	BMNH 1965.M.17405	Foot-pad sample	Y	53
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1934.1.1.9136	Foot-pad sample	Y	84
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1934.1.15978	Foot-pad sample	Y	84
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1965.M.17400	Foot-pad sample	Y	53
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1965.M.17412	Foot-pad sample		53
Carduelis flavirostris rufostrigata (Walton, 1905)	BMNH 1965.M.17475	Foot-pad sample	Y	53

Carduelis flavirostris rufostrigata (Walton, 1905)	BMNH 1949.25.3922	Foot-pad sample	Y	69
Carduelis flavirostris miniakensis (Jacobi, 1923)	BMNH 1914.9.3.91	Foot-pad sample		104
Carduelis flavirostris miniakensis (Jacobi, 1923)	BMNH 1965.M.17471	Foot-pad sample	Y	53
Carduelis flavirostris miniakensis (Jacobi, 1923)	BMNH 1912.9.23.167	Foot-pad sample		106
Carduelis flavirostris rufostrigata (Walton, 1905)	BMNH 1948.27.602	Foot-pad sample	Y	70
Carduelis flavirostris altacia (Sushkin, 1925)	BMNH 1965.M.17469	Foot-pad sample	Y	53
Carduelis flavirostris korejevi (Zarudny & Härms, 1914)	BMNH 1965.M.17465	Foot-pad sample		53
Carduelis flavirostris korejevi (Zarudny & Härms, 1914)	BMNH 1906.12.9.152	Foot-pad sample	Y	112
Carduelis flavirostris korejevi (Zarudny & Härms, 1914)	BMNH 1906.12.9.149	Foot-pad sample		112
Carduelis flavirostris brevirostris (Bonaparte, 1865)	BMNH 1907.12.21.472	Foot-pad sample	Y	111
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1907.12.21.470	Foot-pad sample		111
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1949.Whi.1.9296	Foot-pad sample	Y	69
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1949.Whi.1.9294	Foot-pad sample		69
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1897.12.10.283	Foot-pad sample		121
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1897.12.10.286	Foot-pad sample		121
Carduelis flavirostris brevirostris (Bonaparte, 1855)	BMNH 1887.6.1.320	Foot-pad sample		131
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1934.1.1.8364	Foot-pad sample	Y	84
Carduelis flavirostris pipilans (Latham, 1787)	BMNH 1965.M.17443	Foot-pad sample	Y	53
Carduelis flavirostris flavirostris (Linnaeus, 1758)	BMNH 1965.M.17459	Foot-pad sample	Y	53
Carduelis flavirostris rufostrigata (Walton, 1905)	BMNH 1946.7.43	Foot-pad sample		72

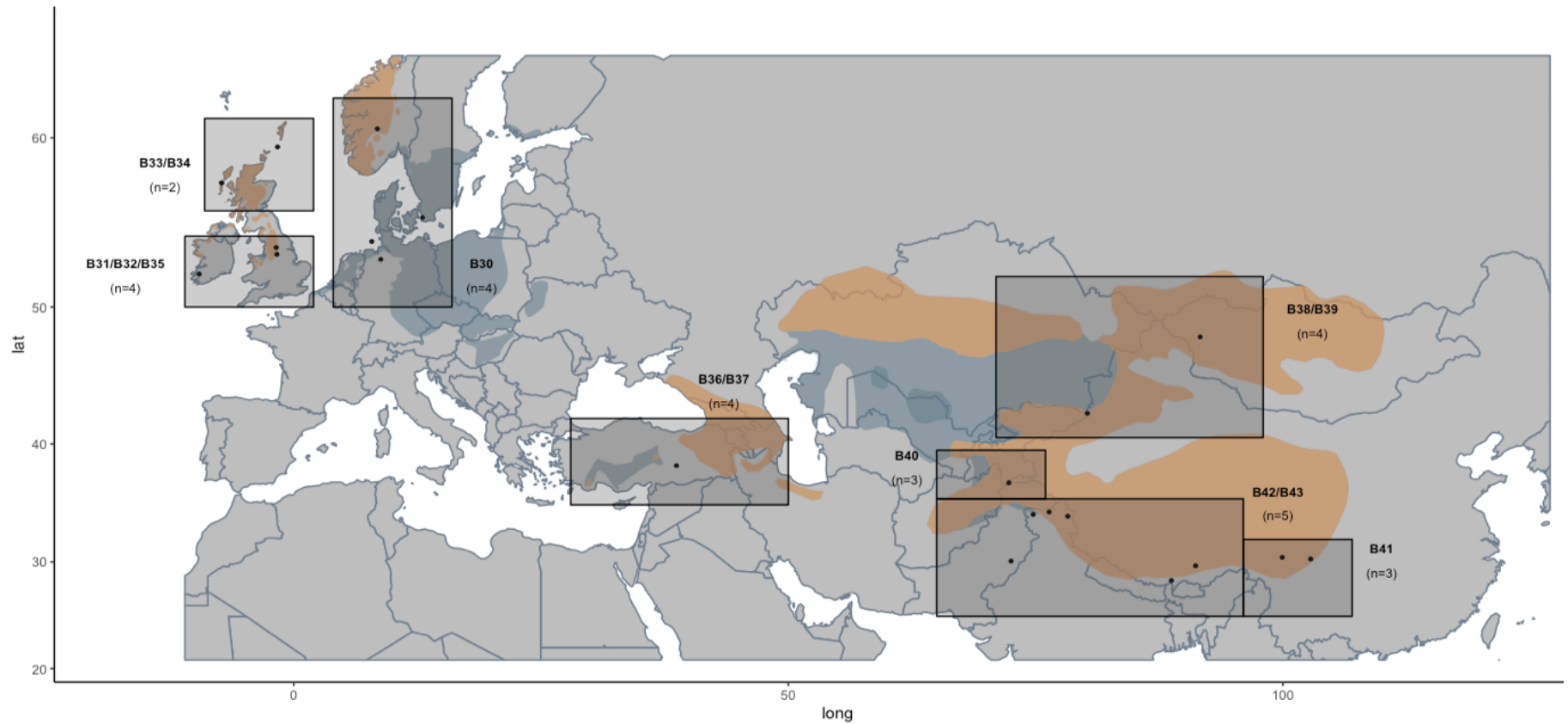


Figure 4.1 Sampling locations of requested samples from the NHM, Tring. Note that each point represents a collection point and the surrounding grey box shows the approximate region for each sampled subspecies within the collection. The number adjacent to each shaded region (i.e. B33/B34) reflects the drawer number in which specimens are stored within the collection.

Gel electrophoresis was carried out under the same protocol as described in chapter 3. In order to amplify a smaller segment of the apparently fragmented COI region of the mtDNA, brand new primers were designed. These primers were designed manually within a known sequence and using aligned sequences to suggest SNP sites on BioEdit. Because COI is a protein coding gene (Buhay 2009), where possible, primers were selected to start on either the first or second codon within a protein sequence. Primers were designed to cover 20-22 bases (table 4.2). Where possible, C and G constituted 55% of the nucleic acid composition and strings for >4 repeat sequences were avoided. Primers were tested against well-established successful sequences (sample: RSPBA, BirdF primer) before running on museum specimens. Of these five primer pairs, one pairing, Twite-Fo5, Twite-Re5 was used frequently. Each primer was tested on a 1% agarose gel with a positive (RSPBA) and a negative (H₂O).

Table 4.2 Primers designed for amplification of smaller segments of fragmented DNA, of which Twite-Fo5, Twite-Re5 were used successfully.

Name	Gene/Region	Primer sequence 5'-3'
Twite-Fo1	Cytochrome c oxidase sub-unit I	TAT ACC TAT TTT CGG CCA TGA GC
Twite-Re1	Cytochrome c oxidase sub-unit I	GAT GTA GTA GGA GAG GAA GGA T
Twite-Fo2	Cytochrome c oxidase sub-unit I	AGC CCC ACA TAG CAT CCC AC
Twite-Re2	Cytochrome c oxidase sub-unit I	GAG GGA GTA GAA TAC TGC AGT AT
Twite-Fo4	Cytochrome c oxidase sub-unit I	TCT GCA TCG CTA AAT CCT T
Twite-Re4	Cytochrome c oxidase sub-unit I	TGA TTG CAC CTA GGA TTG AGG A
Twite-Fo5	Cytochrome c oxidase sub-unit I	TTC GGA AAC TGA CTA GTT CC
Twite-Re5	Cytochrome c oxidase sub-unit I	GGC TAT ACT GTT CAG CCT GT
Twite-Fo6	Cytochrome c oxidase sub-unit I	ATY GTT TYT GGT TRA TTA TTG
Twite-Re6	Cytochrome c oxidase sub-unit I	GTT ACG GTC TGT AGA AAC CA

Gel electrophoresis was run against a 100bp ladder, and as with the primary samples, an equivalent amount of loading dye. The amount of DNA sample was upped slightly from 4µl to 6µl in order to increase the detection of smaller and faded bands on the gel. In order to ascertain if age of sample is linked to fragment size and subsequent sequence success rates approximate ages were calculated for each sample. Museum

accession dates were used to establish a minimum age for each specimen, i.e. if a sample was accessioned into the collection in the year 2000, it must be at least 18 years old in 2018.

Welch's two sample t-test was carried out in base R to explore the relationship between age of specimen and successful amplification. Specimens were grouped into amplification success and years since accession into the collection compared.

Wing length measurements of NHM specimens

In addition to the samples which were taken for DNA extraction and sequencing, where possible, wing cord measurements were taken from Twite specimens within the collection. 250 measurements were taken from eight subspecies, four *altaica*, 13 *brevirostris*, 15 *flavirostris*, 36 *korejevi*, seven *miniakensis*, 50 *montanella*, 62 *pipilans* and 63 *rufostriata*. Measurements were taken using a dedicated metal wing ruler which measures from the carpal joint to the wing point of the closed wing. Several specimens were omitted from these measurements as they were missing either primary feathers which make up the wing point or the wing in its entirety.

An ANOVA test of variance was carried out on all of the wing length data collected, and t-tests for specific pairs of subspecies groups. Only groups with $n \geq 15$ were selected for t-test analyses. Both tests were carried out in base R, ANOVA results are shown in table 4.3.

Results

Wing measurements

Wing measurements were taken from 250 specimens in the NHM collection in order to explore phenotypic differences against genetic results. Significant differences in wing length were found between subspecies of Twite, (ANOVA, $F = 17.99$, $df = 242$, $p = < 0.00001$), see table 4.4. There were also significant differences found between the wing lengths within the European groups where $n = > 15$ (*flavirostris* & *pipilans*), ($t = -3.0548$, $df = 25.732$, $p = 0.005$). Between the Asian groups, *rufostrigata* was found to have a significantly longer wing than the other two subspecies from this region (*montanella* & *korejevi*) ($t = -9.6706$, $df = 123.84$, $p = < 0.00001$), however overall differences between the European and Asian groups were insignificant ($t = 1.7291$, $df = 188.63$, $p = 0.08$). The subspecies *rufostrigata* has a significantly longer wing than all other groups combined ($t = -9.2196$, $df = 106.01$, $p = < 0.00001$).

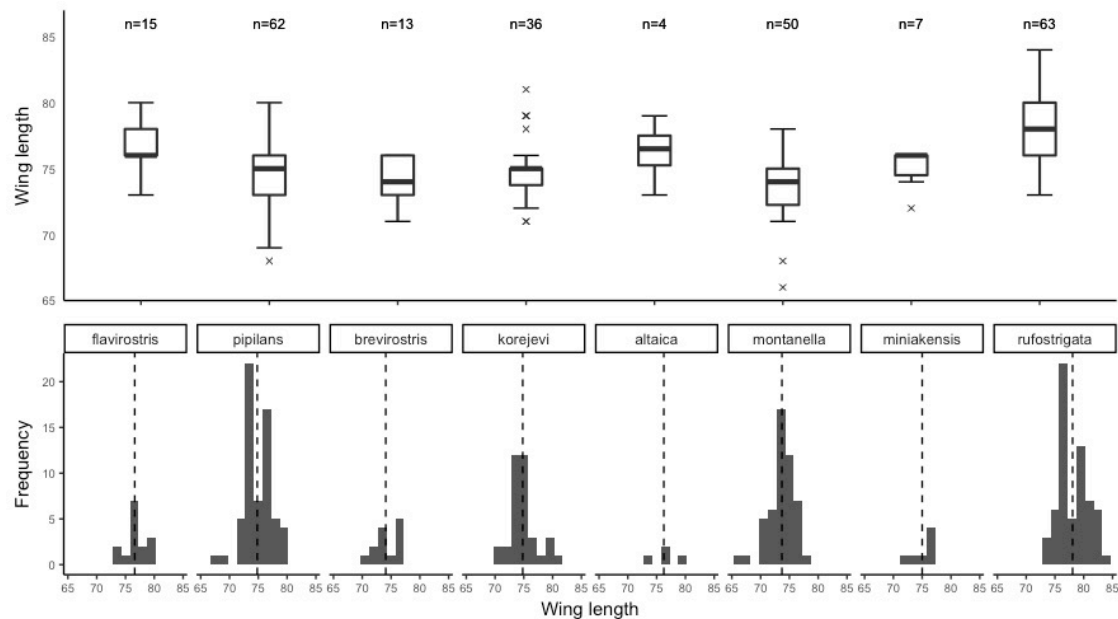


Figure 4.2 Wing lengths of Twite specimens held in the NHM collection, Tring. Top shows summary box plots of the data presented below. Dotted lines show the mean length for each subspecies group.

Table 4.3 Sample size (n), mean and standard deviation of wing lengths for each group of Twite for which n = >15.

	<i>Flavirostris</i>	<i>rufostrigata</i>	<i>pipilans</i>	<i>montanella</i>	<i>korejevi</i>
N	15	63	62	50	36
Mean	76.6	78	74.8	73.7	74.8
SD	1.9198	2.5519	2.3989	2.1969	2.2145

Table 4.4 Results of ANOVA for all samples, including those of low sample sizes (*brevirostris*, *miniakensis*).

ssp	SD	error	t value	Pr(>t)
<i>brevirostris</i>	-2.173	1.319	-1.647	0.1
<i>flavirostris</i>	0.350	1.298	0.270	0.7
<i>korejevi</i>	-1.44	1.298	-1.188	0.2
<i>miniakensis</i>	-1.259	1.446	-0.864	0.3
<i>montanella</i>	-2.550	1.199	-2.127	0.03
<i>pipilans</i>	-1.427	1.190	-1.199	0.2
<i>rufostrigata</i>	1.813	1.190	1.524	0.1

Custom primers

Of the five primer pairs designed to amplify short segments of mtDNA from ‘ancient’ specimens one, Twite Fo5 (TTC GGA AAC TGA CTA GTT CC), Twite Re5 (GGC TAT ACT GTT CAG CCT GT) was successful. This primer pairing amplified 104 bp of COI mtDNA in a region which holds informative markers. Against a known sequence from genebank (GU571794.1) this region within the COI begins at position 243 and terminates at position 355; positions 332 and 338 are informative sites for identification of the Qinghai-Tibetan (*rufostrigata*) group and 262 seems to be variable within the European group.

NHM samples

Ancient DNA samples were aligned against contemporary primary DNA sequences (see chapter 3). The short segments (104 bp) from the COI region of the mtDNA contained an apparently high number of polymorphisms, highly suggestive of extraneous DNA within these samples. The extent to which contaminant polymorphisms occurs varied between sequences; haplotype 1 contained a positive control (RSPBa) as well as a sample from the NHM collection (NHM978) and had no unexpected polymorphisms; likewise, NHM471 corresponds to an Asian control from the Qinghai-Tibetan plateau. The apparent SNPs present in these sequences are otherwise unknown in Twite mtDNA, implying that another species may be the origin of extraneous DNA. A alternative explanation could be that the primers are amplifying nuclear copies following transposition of mtDNA sequences into the nuclear genome (nDNA) (Sorenson and Fleischer 1996).

A neighbourhood joining tree was generated to visualise relationships between ancient Twite samples and other closely related species (figure 4.3). Species with a similar mtDNA signature were chosen based on National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) results. Although the presence of extraneous DNA meant that these samples are unsuitable for alignment against primary samples, small regions of informative loci exist in these data; position 332 in COI

alignments is an informative site for *rufostrigata* with all Tibetan samples showing a cytosine (C) base in this position, where European and Central Asian groups show thymine (T). A second informative position on the COI mitochondrial genome is 338, where *rufostrigata* have an adenine (A) base, where all other groups have a thymine (T), again, this is apparent in all NHM samples.

In addition to the NJ tree, a haplotype network was built to show genetic differences between the samples which amplified successfully. Here the pale green nodes are known Twite samples, one from the UK and a second from Asia. The same species as displayed on the tree as identified by NCBI BLAST results are added in dark green and the NHM samples in pink. Node are generally representative of single frequencies, although two nodes show NHM samples which were consistent with known Twite samples and therefore have a frequency of two. A Welch two sample t-test found a small significant relationship between age of samples (> 100 years) amplified successfully and those which failing to amplify ($t = -2.512$, $df = 17.43$, $p = 0.02$).

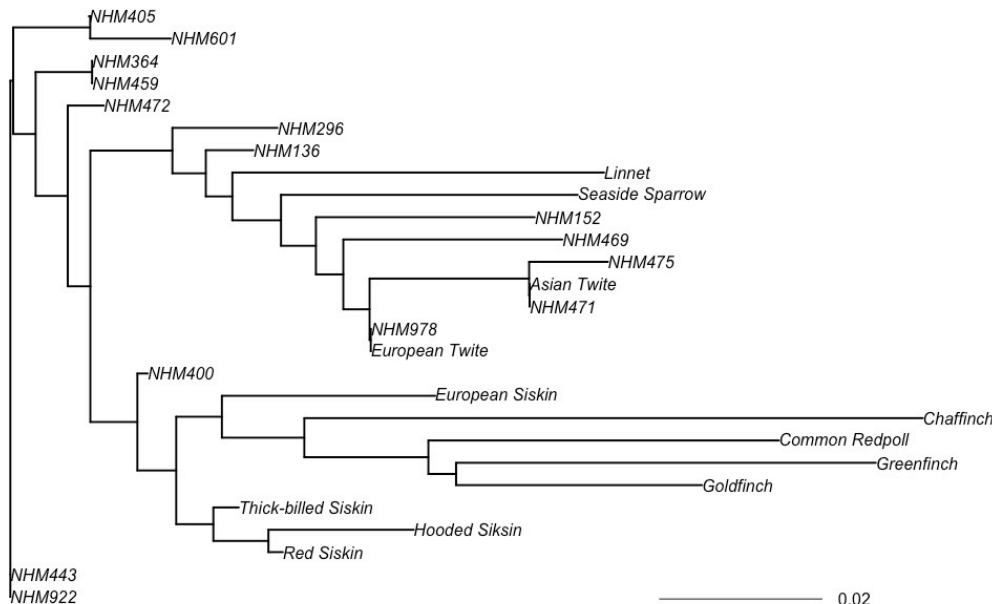


Figure 4.3 Neighbourhood joining tree showing NHM samples, as well as putative contaminant DNA as identified by the NCBI BLAST tool.

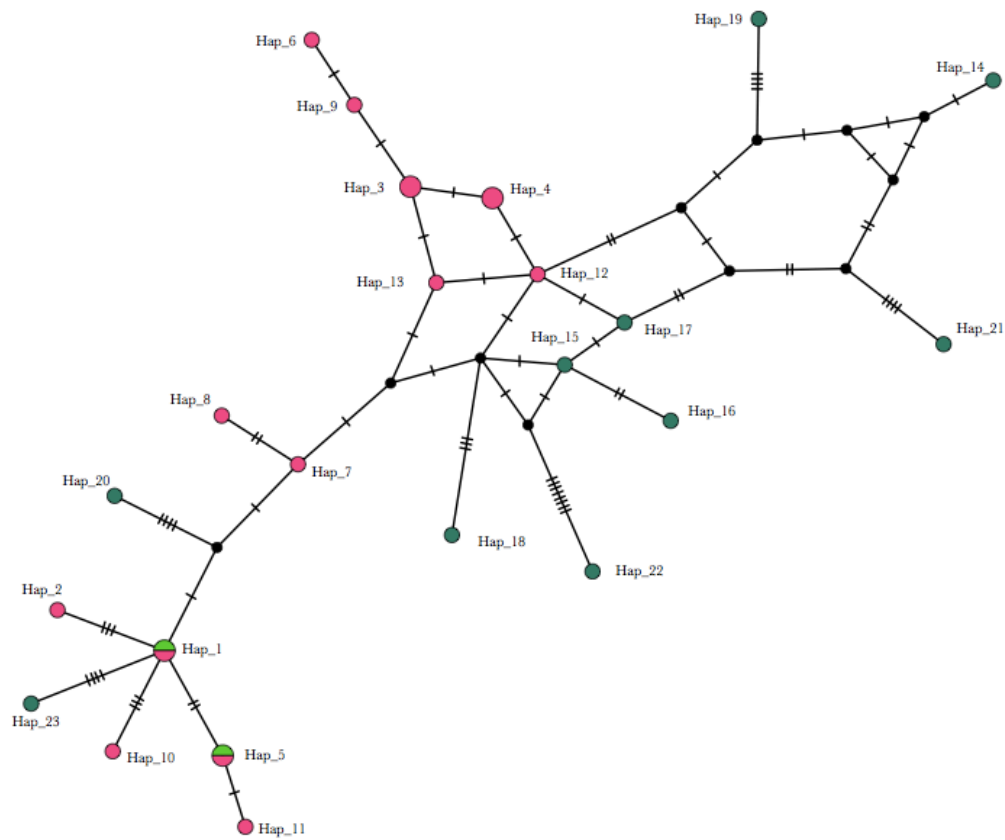


Figure 4.4 Haplotype network showing NHM samples (pink), as well as putative contaminant DNA (teal) as identified by the NCBI BLAST tool. Here two known Twite COI mtDNA sequences in pale green (an Asian and a European) match with two of the NHM samples, see table 4.5.

Table 4.5 Index to haplotypes as identified by the Haplotype network for NHM samples and putative NCBI BLAST identified contaminant species.

Haplotype number	Haplotype frequency	Constituent sequences
1	2	European Twite, NHM978
2	1	NHM152
3	2	NHM443, NHM922
4	2	NHM459, NHM364
5	2	Asian Twite, NHM471
6	1	NHM601
7	1	NHM136
8	1	NHM296
9	1	NHM405
10	1	NHM469
11	1	NHM475
12	1	NHM400
13	1	NHM472
14	1	Goldfinch
15	1	Red Siskin
16	1	Hooded Siskin
17	1	Thick-billed Siskin
18	1	European Siskin
19	1	Common Redpoll
20	1	Linnet
21	1	Greenfinch
22	1	Chaffinch
23	1	Seaside Sparrow

Discussion

Due to the unexpected polymorphisms encountered in the mtDNA of the NHM samples, this study was unable to resolve the relationships between all eight of the sampled subspecies. The origin of this extraneous DNA remains unknown although contamination is likely based on the occurrence of bp differences outside of those expected for the COI region of the Twite mtDNA. Another explanation may be the transposition of mtDNA into the nDNA which is amplified by our primer pair. Standard well-established primers failed to amplify the mtDNA present in the NHM samples due to the fragmentation of the mtDNA present.

Measurements of Twite within the NHM collection revealed that *rufostrigata* is significantly longer winged than its conspecifics. There is little significant difference between all other groups. This observation is common among subspecies which occupy a higher altitude or colder climate than conspecifics (Salewski and Watt 2017).

Size variation between groups

Because these data were collected from museum specimens, wing cord is the only reliable metric of size post preparation; age and sex data can be misinterpreted, weight can be unreliable and is seldom recorded and total length can be biased depending on preparation method (Knox and Walters 1992). Within several Twite subspecies, wing length follows a bimodal distribution which is consistent with size discrepancies between male and female birds (Clark and Sellers 2001). Within the wing cord data, the subspecies group *rufostrigata* has a significantly longer wing than other Twite groups. The European *flavirostris* is also longer winged than most, but these measurements were not significant. The latter corresponds to this group occupying the highest breeding latitude ($\sim 58^{\circ}$ - 68°) of any Twite group, extending into the arctic circle. Conversely, the Tibetan *rufostrigata* occupies the lowest breeding latitude of any Twite group ($\sim 28^{\circ}$) yet has the longest wing, however this group also occupies the highest altitude, with a mean of ~ 4500 m above sea level on the Qinghai-Tibetan plateau (Qu et al. 2010). This follows Bergmann's rule that within species and amongst closely related

species of homoeothermic animals, a larger size is often achieved in colder climates than in warmer ones, which is linked to the temperature budget of these animals (Salewski and Watt 2017), however, body size is also driven by interactions between the physiological demands of the environment and resource availability (Orme et al. 2009), of which there is little data for these groups.

mtDNA from NHM samples

The cause of the unexpected polymorphisms in the NHM Twite sequences remains unknown. Rapid mitochondrial divergence or introgressive hybridisation are unlikely causes based on both the extent across all groups and the seemingly random pattern of occurrence across the sequenced region (Burton, Ellison, and Harrison 2006; Zheng et al. 2011). Although as it affects almost all of samples and most polymorphisms are inconsistent with known Twite mtDNA, and although all precautions were taken at both the sampling (from museum specimens) and DNA extraction stages, extraneous DNA is the most probable cause. Several sequences showed polymorphisms consistent with their geographic origin (see all NHM *rufostrigata* samples) and a further two had no unexpected polymorphisms at all; The difference in polymorphism ranged from 0 (NHM978 & NHM471) unexpected polymorphisms to 7 (NHM601) in the 104 bp fragment.

A significant relationship was found between the age of samples and PCR amplification success, two of nine (~22%) samples >100 years old were successfully amplified where 13 of 17 (~23%) <100 years old were successful. Samples for which PCR amplification was not possible were considered to be too heavily fragmented to be viable for this study. Age could possible account for some of the extraneous DNA however; samples were taken from the underside of the toe-pad (see chapter 4: methods) of dry stored Twite skin specimens and therefore extraneous DNA could theoretically exist on the outer layers of the skin. This is especially true where specimens have historically been stored with other species. The bird collection of over one million skins has been moved on several occasions during its history (Knox and Walters 1992) and there is no detail

about the condition of passage from initial collection site to the museum where not already provided.

Although less likely, nuclear mitochondrial transposition into the nDNA may also explain unexpected polymorphisms in the mtDNA. This natural transfer of mtDNA to the nDNA creates nuclear copies or a nuclear mitochondrial DNA segment (NUMTs) (Hazkani-Covo et al. 2010). Mitochondrial pseudogenes were once considered infrequent and short in the avian genome (Pereira and Baker 2004), but recent studies have found that NUMTs are large and common in at least the falcons (Nacer and Raposo do Amaral 2017). There is no data on the frequency of NUMTs in the passerines specifically.

Chapter 5

Conclusions and implications for future conservation

General conclusions

This study set out to address the extent to which population disparities at differing scales affect within-species connectivity between Twite groups globally. Twite were ringed across Europe between 2013 – 2018 with additional data accessed from the EDB and NSBC. Within the European group, ringing data analysis appears to contradict molecular results. Migratory connectivity in this region suggested by this study found that British Twite form discrete non-breeding groups with high site fidelity year on year, which is consistent with previous findings (Raine 2006a). The Scandinavian group however, differ markedly, they are more weakly connected to winter grounds (Dierschke 2002) with high vagility and greater migratory plasticity over the non-breeding season. This is likely to be an adapted response to environmental uncertainty, see Blackburn et al. (2017), and may suggest a higher tolerance for environmental change than the British population.

Although ringing records of birds crossing between the two groups are rare, contact is most likely to occur along either shore of the North Sea coast, where birds are in close proximity during the non-breeding season. Dutch bird atlas data has suggested that Scandinavian flocks wintered at higher latitudes and in lower numbers between 2012 – 2016 than they did during the same months between 1979 – 1983 (Sonvon 2016). This means that continental Twite now rarely extend as far south as the English Channel coast, and so, may be preventing recruitment over winter. Although little quantifiable evidence is available to support claims that continental Twite groups have shifted over time, Shorelark, which associate strongly with Twite over winter (Dierschke 2002) are recorded as have shifted west to North Sea coasts in recent decades (Sutherland 1998), perhaps related to climatic change.

There are no comparable ringing data for the Asian Twite, and as a result the limits and distributions of subspecies within this region are poorly understood (Cramp and Perrins 1994; Clement et al. 201; Wassink 2015). However, there are no records of western European Twite recovered in Asia or Asia minor and no Twite in Europe were recorded as belonging to any of the morphologically distinct Asian subspecies. Ring/recovery records between groups (be those within Europe or internationally) are rare.

The apparent isolation between British and continental groups was not reflected in the molecular data, which found a single European haplogroup. None of the mtDNA haplogroups identified by this study aligned with currently accepted subspecies within the European Twite distribution. The finding of a single European haplogroup suggests that gene-flow exists, or existed until very recently, between the two groups in this region. This study found population structure in between the European and central Asian groups north of the Himalayas and extending as far east as Inner Mongolia. These central Asian birds are genetically distinct but still closely related to the European group. The population of Twite which occupy the Tibetan plateau is highly divergent from neighbouring central Asian Twite despite geographic proximity, as first suggested by Qu et al (2010), and corroborated by this study.

Molecular data on Marsh tit was also accessed in order to compare with Twite, as both have a global disjunct population. Within the Marsh tit out-group, European birds are closely related to those across the disjunction, in Central and east Asia. Genetic differences here may be explained by mitochondrial introgression between Marsh and Black-bibbed tit *Pocile hypermelaenus* in the eastern Palearctic (Tritsch et al. 2017).

Rarely have the phylogeographic consequences of population disjunction been addressed in Palearctic passerines and so, comprehensive pan-species analysis would be valuable. However, the results presented by this study suggest that the disjunct distribution in Twite is not the most formative aspect to population structure, but more likely vicariance forming a barrier between the Asian groups, probably linked to the formation of the Himalayas.

These findings also suggest that the closely related European and central Asian groups radiated out from the Tibetan plateau prior to, or during the Pleistocene epoch. It seems likely that glacial processes shaped both the global distribution of Twite but also, the genetic relationships between identified haplogroups. Additional sampling within the Asian distribution would help to corroborate this theory and define population limits around the Himalayan steppe and central Asia.

Twite population genetics

This study finds three clusters of haplotypes (or haplogroups) based on sequenced mtDNA; One haplogroup contains the Western European birds, including *pipilans*, *flavirostris* and the relict *bensonorum* of northern Scotland, there are no informative sites for these constituent populations within this haplogroup however a number of haplotypes have been identified within the region. A second central Asian Twite haplogroup in which five haplotypes have been identified comprise of at least the subspecies *altacia*, and probably *brevirostris* and *korejevi*. The third haplogroup contains the divergent Tibetan sub-specific group *rufostrigata*, which is represented by several haplotypes. Qu et al. (2010) reported mixing between regions within the Qinghai-Tibetan plateau, but no data has been made available to infer subspecies. The affinities between the latter two groups are unclear due to restrictions in available data from Asia, however Twite which live in the Qinghai-Tibet region are markedly different from those found across the mid latitudes to the north and west. Population structure exists between the three haplogroups and F_{st} suggests at least historic gene-flow between all three groups. Haplotype networks constructed during the course of this study found that despite close proximity, the central Asian birds only differed from European Twite by two bps, but had a nine bp difference from those breeding in the Qinghai-Tibetan plateau.

IUCN classification and subspecies limits

The current international IUCN classification of Least Concern (LC) treats Twite as a single global species group, whilst a European regional assessment (EU27), which includes Asia minor, find Twite to be vulnerable (VU) to extinction. This study suggests that the data used to calculate these classifications may be deficient in both subspecies limits and population trends for much of the global range. The declining western European group should be treated separately within the regional assessment, to better reflect the parapatric distribution and differing ecology from the wider population and in order to protect the genetic diversity within the region. Additionally, perhaps the exclusion of the divergent Tibetan group(s) from the Twites international IUCN status would better reflect the protection of genetic and ecological differences identified within the region.

Tobias et al (2010) suggested quantitative criteria for species delimitation in order to overcome taxonomic biases and uncertainty in the fields of biology, ecology and conservation based on divergence between undisputed sympatric species. Following the proposed magnitude scoring system, the *rufostrigata* subspecies of Twite from the Tibetan plateau score close to that required for species delimitation. Such future delimitation either through elevation to species status or in the acknowledgment of a super-species group would affect the IUCN status and subsequently the conservation of the species as a whole. It is not within the scope of this study to suggest species delimitation between Twite groups but molecular, behavioural and morphological traits, apparent particularly between the *rufostrigata* and remaining groups warrant further attention.

Implications for conservation management

Demographic imbalances

Low connectivity and recruitment between breeding groups, such as those found by this study, may perpetuate demographic imbalances, which are more apparent in smaller populations. Gruebler et al (2008) show that demographic imbalances (such as sex ratio, or low juvenile survival) in small passerine populations increase the rate of decline. The small and isolated cliff nesting (Frost 2008; Dunning et al. 2016) population of Twite in north Derbyshire had an apparent breeding season (male/female) adult sex ratio of 1.6:1 ($n = 29$) in 2017 and 3:1 ($n = 27$) in 2018. The surplus of males in breeding condition results in unusually high levels of intraspecific aggression at supplementary feeding stations and prevents conspecifics accessing resources. Sex ratio imbalances are common in birds, particularly in non-passerines; for example, the growing sex ratio bias in dabbling and diving ducks (Fox and Cristensen 2018) is thought to be the result of increased female vulnerability during the nesting period, of which the males take no part (Sargeant, Allen, and Eberhardt 1984). This is corroborated by sex ratio parity in first winter (and therefore pre-nesting age) ducks (Mitchell et al. 2008). Like Twite, most dabbling and diving duck species are ground nesters, preferring low vegetation and open space, with females tending the nest. Females which nest in meadow and low vegetation environments may face new anthropogenic pressures from terrestrial predators, changing farming practice and over-grazing (Chamberlain et al. 2000; Newton 2013).

Whinchat *Saxicola rubetra* are a meadow nesting passerine occupying a similar breeding niche to Twite. Whinchat adult survival over five day periods was found to drop during periods of agricultural mowing, resulting in 80.6% of males and only 68.4% of females surviving the breeding season, anthropogenic female mortality resulted in a local population decline 1.7 times faster than the background rate (Gruebler et al. 2008). Within the passerines more generally, adult sex ratios in wild birds remain poorly described, but male biases are common, particularly in threatened species (Donald

2007) and those from small or fragmented populations (Fretwell and Lucas 1970; Zanette 2001). These imbalances can be driven by mortality, but also demography factors (i.e. sex-biased juvenile dispersal). Small populations are more likely to have skewed sex ratios (or other demographic imbalance) if survival or demographic factors work to a greater extent on one sex or age class. Such demographic specific sex ratios were found to vary greatly in a passerine well distributed around Britain and Ireland, the Willow warbler *Phylloscopus trochilus*, with the highest levels of male bias in those areas of lowest overall abundance; these male-biased sex ratios were found to affect localised reproductive output (Morrison et al. 2016).

Provisional survival analysis has been carried out on the English Twite groups using data collected by this study and others between 2002 - 2017 (Wilkinson & Dunning, in prep, 2018). However, these figures are based on low sample sizes and carried out over short time periods. A more thorough analysis of the data collected and stored in the EDB, throughout western Europe would allow for more detailed conclusions to be drawn between survival and demographic factors such as migratory connectivity.

Limitations to monitoring and future research

Twite breed at low frequencies across large areas of remote homogenous habitats, such as the moorland, steppe and sea cliffs. This breeding ecology make Twite a difficult species to study during the breeding season, as is reflected in the winter bias of EDB ringing records and the lack of available ringing records from central and eastern Asia. Colour marking, as used by this, as well as previous studies (see, Raine 2006a; Atkinson 1998), has found that Twite in Europe have a high breeding and foraging site fidelity and so post-breeding flocks can be reliably targeted for specific research.

Passive monitoring methods such as colour marks are limited by the distribution of observers to report marked individuals, which creates a bias in recovery data towards areas with good public access and close to infrastructure (Du Feu et al. 2016).

A clear limitation to this study was the lack of ringing data from the Asian breeding Twite groups, however this is not easily overcome without considerable investment into avian

research in remote areas of the region. Instead, focus on describing the ecology and distribution limits of these groups would be valuable to identify further conservation priorities. This study attempted to investigate gene flow across the Twites global range, however samples from certain populations were unobtainable, creating gaps in the data. Particularly under-represented was the *brevirostris* & *korejevi* groups of Asia minor and the Caucasia respectively. Recent distribution maps have suggested mixing between the two groups although evidence for this is weak (Shirihai and Svensson 2018). Data from central Asia is only represented here by a very small sample size, which makes up a haplogroup covering a large geographic area, and more comprehensive sampling of this area is needed to explore relations between haplotypes and current subspecies limits.

This study made little attempt to quantify phenotypic traits between groups such as voice, plumages or morphology (beyond brief notable plumage differences and wing cord measurements, see chapters 3 & 4). However, work of this type can complement molecular data to suggest species or group delimitation (Tobias et al 2010). To this end, spectrophotometry measurements of plumage and analysis of call/song recordings to address proposed differences in voice (Zablotskaya 1978) would be useful. This study found strong indicator of divergent morphology and niche filling capability associated with delimitation. (Tobias et al. 2010).

As well as additional DNA sampling and phenotypic difference analysis, future work should attempt to sequence a larger region on the genome. A study comparing mtDNA and microsatellites as indicators of isolation by distance (IBD), found that mtDNA is often in conflict with multi-locus markers (such as microsatellites). This is likely the result of a selection-driven reduction in genetic diversity (Teske et al. 2018), so any work which seeks to corroborate the findings presented here would benefit the inclusion of nuclear DNA markers as well as additional regions of the mtDNA (such as ND2 & d-loop).

Recommendations for conservation

The findings presented here suggest that, the western European Twite should be treated as an isolated group from the rest of the Asian population. This includes those represented in Asia minor, which under the current IUCN EU27 regional assessment they are not. In addition to this the Qinghai-Tibetan plateau population are highly divergent from the central Asian, which should also be considered in regard of future research and conservation classification. However, further research is required in this region to better understand spatio-temporal relations between the Asian groups, particularly those to the north and east of Tibet. This Improved understanding should then inform the current data deficient international IUCN classification for Twite.

Within Britain, where natal origin informs winter site selection, additional conservation measures should be implemented into the non-breeding distribution of Twite, which lack the plasticity of their continental kin. This should include revisiting non-breeding distributions across western Europe (largely neglected since Atkinson 1998), in order to identify the current distribution.

Twite specialise on pioneer mud flat (Atkinson 1998; Dierschke 2002a) before ecological succession growth prevents foraging on the ground. Habitat destruction along the Wadden sea coast in the 1960's caused a temporary dip in wintering Twite, Snow Bunting and Shore lark, which recovered following the recovery and expansion of salt marsh habitat in the 1980's (Dierschke 2002a). Along the British coast habitat re-classification work of this nature should now be high priority following Atkinson (1988).

Limited gene flow between groups could, in time erode the ability of Twite to adapt to environmental change and therefore consideration should be made to how such factors affect the habitat and phenology of populations. Regardless of spatial scale, Twite are a species defined by fragmentation and disjunction, and therefore threats may be population specific. As well as the disparities in connectivity and molecular structure presented by this study, it is clear that localised, group specific research is required for conservation of this species to be successful.

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