Stable isotopes and mtDNA reveal niche segregation but no evidence of intergradation along a habitat gradient in the lesser whitethroat complex (*Sylvia curruca*; Passeriformes; Aves)

Stephen C. Votier[1] s.c.votier@exeter.ac.uk
Simon Aspinall[2]
Stuart Bearhop[3] s.bearhop@exeter.ac.uk
David Bilton[4] D.Bilton@plymouth.ac.uk
Jason Newton[5] j.newton@suerc.gla.ac.uk
Per Alström[6] per.alstrom@slu.se
Paul Leader[7] pjleader@asiaecol.com.hk
Geoff Carey[7] gjcarey@asiaecol.com.hk
Robert W. Furnes[8] Bob.Furness@glasgow.ac.uk
Urban Olsson[9] urban.olsson@zool.gu.se

[1] Environment & Sustainability Institute, [3]Centre for Ecology & Conservation, University of Exeter, Penryn Campus, Cornwall, UK TR10 9EZ, UK
[2] Deceased
[4] Marine Biology and Ecology Research Centre, University of Plymouth, Plymouth PL
[5] NERC Life Sciences Mass Spec. Facility, SUERC, Rankine Avenue, East Kilbride, Glasgow G75 OQF, UK
[6] Department of Animal Ecology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18 D, SE-752 36 Uppsala, Sweden
[7] Asia Ecological Consultants Ltd, 127 Commercial Centre, Palm Springs, Hong Kong.
[8] College of Medical, Veterinary and Life Sciences, Graham Kerr Building, University of Glasgow, G12 8QQ, UK
[9] Department of Biology and Environmental Science, University of Gothenburg, Box 463, SE-405 30 Gothenburg, Sweden

Abstract

Niche segregation plays a critical role in the speciation process, but determining the extent to which taxa are geographically or ecologically isolated is challenging. In this study we use stable isotopes of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), hydrogen ($\delta^{2}H$) and oxygen ($\delta^{18}O$) to test for ecological differences among taxa in the Lesser Whitethroat *Sylvia curruca* complex. Analysis of mitochondrial DNA (mtDNA) revealed 6 distinct haplotype groups, which conform to at least 5 distinct taxa. Stable isotopes provided insight into geographical and broad-scale ecological differences among haplotypes. The most striking isotope differences were between the populations inhabiting Siberian boreal forest (*S. c. blythi*) from the one inhabiting semi-desert in Kazakhstan (*S. c. halimodendri*). It is generally assumed that these two populations form a morphological cline along a gradient from mesic to xeric habitat. Our sample includes a large proportion of morphologically intermediate individuals that appear to represent a hybrid population. However, in all of these there is strict correspondence between haplotype and isotope signature, suggesting an ecological division on the breeding grounds between all our samples of these two taxa. The lack of ecologically intermediate individuals among our sample of morphologically intermediate ones thus speaks against the existence of a cline. The two taxa *blythi* and *halimodendri* emerge as potential models for the study of the early stages of the speciation process. While differences in stable isotopes may be largely influenced by geography, we also demonstrate how, in specific instances (such as the alleged cline reported here) may be used to evaluate niche segregation between taxa, providing information of importance for determination of species limits.

Key words: $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$, phylogeography, speciation, warbler, *Sylvia curruca*, cline, stable isotopes

Introduction

Speciation generally involves a three-step process – range fragmentation, the development of reproductive isolation between spatially separated populations, followed by range expansions leading to sympatry (Price, 2008; Mayr and Diamond, 2001). To be able to coexist in sympatry, reproductive isolation between genetically distinct taxa is required to avoid introgression (Endler, 1977; Mayr and Diamond, 2001; Price, 2008), as are probably also ecological differences (Chesson, 2000; Mayr and Diamond, 2001; Price, 2008; Price et al., 2014). In birds there is evidence that it may take more than two million years for reproductive isolation to be completed (Price, 2008; Weir and Price, 2011).

The Lesser Whitethroat complex (*Sylvia curruca sensu lato*, Sylviidae, Passeriformes, Aves) is a group of morphologically similar insectivorous warblers, breeding across almost the entire Palearctic, from Western Europe to east Siberia, and southwards through northwestern China and Central Asia to south-western Iran (Cramp 1992, del Hoyo 2006, Mayr 1986, Olsson *et al.* 2013, Shirihai *et al*. 2001, Vaurie 1959) (Fig 1). The subtle morphological variation between the different taxa in this complex has long obscured the taxonomy, but Olsson *et al.* (2013) proposed, based on analyses of mitochondrial DNA, that it consists of six well supported clades (Fig. 2). Four of these clades, representing *S. c. althaea, S. c. blythi, S. c. halimodendri* and *S. c. margelanica*, occupy more or less parapatric ranges in Central Asia, which share a most recent common ancestor 1.95 $\pm$ 0.55 million years ago (Olsson *et al.,* 2013). Consequently, these clades seem to be mainly below the critical two million year level of divergence, offering one possible explanation as to why they do not occur in sympatry Several authors point out that there seems to be a morphological cline between *S. c. blythi* of the Siberian boreal forests and *S. c. halimodendri* of Central Asian semi-desert (Loskot, 2005; Shirihai *et al.,* 2001; Vaurie, 1959). A morphological cline could arise if reproductive barriers between two previously separated populations are incomplete or break down after they come into secondary contact (Endler, 1973, 1977). Gene flow between the two taxa would dilute the characteristics of the parent

83 taxa, rendering the hybrids phenotypically intermediate to a varying degree. Olsson *et al*
84 (2013) estimated that *S. c. blythi* and *S. c. halimodendri* diverged 1.41± 0.42 million years
85 ago, indicating that they may not yet have reached a stage of divergence where they are
86 able to remain reproductively isolated upon secondary contact (Goldberg and Lande, 2006;
87 Price, 2008; Weir and Price, 2011), making a scenario of secondary gene flow plausible. A
88 morphological cline could theoretically also arise as a result of isolation by distance (Wright,
89 1943), particularly when divergent selection is strong towards the ends of the distribution
90 range due to e.g. differences between habitats or other ecological factors (Endler, 1973,
91 1977). In both cases, individuals in the centre of the cline would be expected to be less
92 habitat specific, and haplotypes typical of one taxon could have spread to individuals being
93 morphologically more similar to, or occurring in habitat more characteristic of, the other
94 taxon.
95
96 Both *blythi* and *halimodendri* have distinctive habitat requirements in the core areas of their
97 respective ranges, but Olsson *et al.* (2013) could not evaluate the alleged cline between
98 *blythi* and *halimodendri* as these taxa occur over a very wide range and in areas that are
99 difficult to access, leading to a paucity of detailed observations on the breeding grounds. In
100 fact, there is a general paucity of records between approximately 50 and 55°N (Lars
101 Svensson in litt.). Dement'ev and Gladkov (1968) list the distribution of both *S. c. blythi* and
102 *S. c. halimodendri* in some detail, and make no mention of intermediate individuals among
103 the few observations from within the contentious area. According to their account, all
104 specimens from north of a line from Yekaterinburg (56°N) to Omsk (55°N) and the
105 Novosibirsk (55°N) and Barnaul (53°N) area are *blythi*, and records of breeding season
106 *halimodendri* are more or less restricted to the south of 50°N. The type locality of *S. c.*
107 *halimodendri* is located relatively close to the apparent northern limit of the taxon at
108 approximately 48°N. Almost all evidence of intergradation stems from a large proportion of
109 specimens of morphologically intermediate appearance that have been collected outside of
110 this area. Furthermore, the morphological similarity between *blythi* and *halimodendri* (cf.

Shirihai et al., 2001) makes some single individuals almost impossible to diagnose with certainty based on morphology alone, particularly considering that an individual exhibiting intermediate characters may be a hybrid.

Unfortunately, diagnosis based on mitochondrial DNA data is almost equally unhelpful when it comes to diagnosing morphologically intermediate individuals. Although mitochondrial haplotype will unambiguously assign an individual to one of the clades identified by Olsson *et al.* (2013), it will not reveal whether an individual that is morphologically intermediate is of hybrid origin or just represent an extreme end of a within-taxon morphological variation. Moreover, Olsson et al. (2013) found no fixed differences between these taxa in nuclear markers. The most likely reason for this is that the time since these lineages diverged is too short for fixed differences to occur in these markers. For these reasons, we sought ways to indirectly collect information pertaining to the ecological requirements and relationships of these two taxa.

The analysis of stable isotope ratios has emerged as a powerful tool for ecological study in recent decades (e.g. Bearhop *et al*., 2004; Boecklen *et al.*, 2011; Bowen *et al.* 2005; Charmantier *et al.,* 2014; Inger & Bearhop 2008; Newsome *et al*., 2007; Post, 2002; West *et al.*, 2006), and, in the context of the present study, may be helpful in resolving ecological differences among the closely related members of the lesser whitethroat complex in general and *blythi*,/*halimodendri* in particular. This approach relies on the fact that naturally occurring gradients in stable isotopes are reflected in consumer tissues in a predictable manner. Some keratinous tissues like hair, feather or nail are metabolically inert following synthesis and so maintain an isotopic record reflecting the location where the tissue was synthesized (Schell *et al*. 1989; Mizutani *et al*. 1990). Moreover, in the case of feathers they provide data covering the period over which they are grown (weeks to months; Bearhop et al. 2003). This time-integrated information on organic carbon sources for heterotrophs and information on habitat of origin may be more informative than observations of habitat unless the latter are

conducting over extended periods.

Here we measure isotopes of hydrogen (δ^2H), carbon (δ^{13}C), nitrogen (δ^{15}N) and oxygen (δ^{18}O) in Lesser Whitethroat feathers grown during the breeding to provide information on food choice and habitat use. The ratio of heavy to light hydrogen ^{1}H: ^{2}H (expressed as δ^2H) and oxygen isotopes ^{18}O:^{16}O (expressed as δ^{18}O) vary largely because of isotopic fractionation during the phase change for vapour to liquid or solid associated with precipitation, with the proportion of δ^2H and δ^{18}O decreasing on a continuous scale with increasing latitude, with distance from the sea and with increasing altitude (Bowen, *et al*. 2005). We here use δ^2H and δ^{18}O primarily as broad-scale markers of distribution within the Lesser Whitethroat complex, as well as to test for altitudinal differences among clades. The ratio of heavy to light carbon isotopes 12C: 13C (expressed as δ^{13}C) in primary producers (and therefore in upper trophic levels too) varies on a discrete scale as a function of different photosynthetic pathways – C3 plants having lower ^{13}C values compared with plants utilising C4 photosynthetic pathways, with plants utilising crassulacean acid metabolism (CAM) being somewhat intermediate but with ^{13}C values usually most similar to C3 plants (Peterson and Fry 1987; Tieszen et al.1983). The ratio of 14N:15N (expressed as δ15N) increases with each trophic level (DeNiro and Epstein 1981), and, although interpretations are not always straightforward (Vanderklift and Ponsard, 2003), may contribute evidence of possible differences in food choice.

Our stable isotope analysis was used primarily to evaluate the existence of a hybrid zone between the morphologically intergrading *S. c. blythi* and *S. c. halimodendri.* This alledged hybrid zone is located in an area difficult to access, and it was not possible to obtain samples from this region. Instead we combined samples from the wintering grounds and all individuals caught during one spring migration season at a locality south of the breeding ranges of both taxa. A basic assumption is that individuals occupying the extensive area

where semi-desert gradually changes into boreal taiga would be exposed to different environmental conditions than individuals living in either semi-desert or taiga. These different conditions would be assumed to produce isotopic signatures rendering populations from within the cline different from populations occupying the extreme ends of the cline. Furthermore, if the apparent morphological cline between these two taxa is best explained by extensive ongoing geneflow across a continuous range, we expect stable isotope signatures from the transitional area to show a lack of correlation to haplotype group. We here test a hypothesis that no hybrid zone exists, and that morphologically intermediate individuals exist for other reasons than geneflow. This hypothesis would be rejected if isotope signatures are not correlated to haplotype group.

Methodology

Taxonomic names and sampling

Throughout we follow the taxonomy according to Olsson *et al*. (2013). 74 of the samples used by Olsson *et al*. (2013) were analysed for stable isotopes (GenBank accession numbers given in Supplemental Table 1). As we were unable to access the area of alleged clinal overlap between *S. c. blythi* and *S. c. halimodendri*, the majority of the samples used for isotope analysis came from birds caught on migration or in the winter quarters throughout Central Asia and the Middle East (Table 1), and were diagnosed based on mtDNA haplotype, so that each of the Central Asian clades identified by Olsson *et al*. (2013) were included. Olsson et al. (2013) demonstrated that haplotypes were in most cases more strongly correlated to breeding ranges than were morphological features. We have thus here adopted the view that the haplotypes are the most reliable taxonomical indicators. All samples of *S. c. blythi* came from south of the area of the alleged cline. We used 19 *blythi* caught on northward migration in Kazakhstan during a period spanning most of the month of May. These samples were all originally identified as belonging to one of the desert forms (i.e.

193 *halimodendri* or *minula*) based on morphology, but were re-identified *a posteriori* as *blythi*
194 based on their haplotype. The bulk of our *blythi* samples may thus be characterised as being
195 either morphological intergrades or *halimodendri* with *blythi* haplotype. Two additional
196 morphologically normal *blythi* from the United Arab Emirates were sampled on the wintering
197 grounds. The migrating or wintering *S. c. halimodendri* included in the study were all
198 originally correctly assigned to one of the desert taxa *S. c. halimodendri* or *S. c. minula*,
199 which may in this context be considered to be the same due to previous taxonomic
200 confusion. We have not come across any individuals with *halimodendri* haplotype showing
201 phenotypic characters typical of *blythi.*
202
203
204 ***Stable isotope analyses***
205 Isotope ratios were measured from tail feathers plucked from birds caught either on the
206 breeding grounds, during the non-breeding season or on migration. Since Lesser
207 Whitethroats undergo a complete post-breeding moult on the breeding grounds (Svensson
208 1992, Shirihai *et al*. 2001), stable isotope ratios of feathers most likely represent breeding
209 habitat preferences. Although a small proportion of Lesser Whitethroats moult tail feathers at
210 other times of the year (Svensson 1992, Shirihai *et al*. 2001), these feathers are younger
211 and less worn than those grown on the breeding grounds, and any such feathers were
212 excluded from our study.
213 Prior to analysis, feathers were washed with water and air-dried, the rachis was
214 homogenised and ~0.7mg was weighed into either a tin cup (for nitrogen and carbon
215 isotopes) or a silver cup (for hydrogen and oxygen isotopes). Analyses were conducted at
216 the East Kilbride Node of the Natural Environment Research Council Life Sciences Mass
217 Spectrometry Facility via continuous flow isotope ratio mass spectrometery (CF-IRMS) using
218 a Costech (Milan, Italy) ECS 4010 elemental analyser interfaced with a Thermo Electron
219 (Bremen, Germany) Delta XP mass spectrometer. For hydrogen and oxygen isotope ratio
220 measurements, a separate ~0.7mg aliquot was weighed into a silver capsule and run by CF-

IRMS on the same instrumentation, but using the Costech HTG-02 reactor (see Newton 2010 for description). Isotope ratios are reported as δ-values and expressed as ‰ according to the equation $\delta X=[R_{sample}/R_{standard})-1] \times 1000$, where X is ^{2}H, ^{13}C, ^{15}N or ^{18}O and R is the corresponding ratio $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{18}O/^{16}O$ and $R_{standard}$ is the ratio of the international references for each element. Hydrogen isotope analysis of feather samples is not straightforward since around a fifth of the hydrogen in keratin can exchange readily with ambient water vapour. We used the comparative equilibration procedure of Wassenaar and Hobson (2000, 2003) and the CFS and BWB-II standards reported there, to correct for non-indigenous hydrogen.

Statistical analysis

To determine whether isotope values varied as a function of haplotype, we used Multivariate Analysis of Variance (MANOVA). The stable isotope values for $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ were included as the dependent variables with haplotype as a six-level factor. In the case of a significant overall model, we then used one-way Analysis of Variance (ANOVA) to determine differences among isotopes and post-hoc Tukey HSD multiple comparisons to identify specifically which haplotypes differed. All data met assumptions of homoscedasticity and normality except $\delta^{18}O$, which was normally distributed following $\log_{10}$ transformation.

Results

Overall there were significant isotopic differences among the six lesser whitethroat haplotypes (Fig. 3, Table 2; MANOVA, Wilk's $\lambda=F_{24,\ 137.3}=4.59$, $P<0.001$, Figure 3) and univariate analysis revealed differences among $\delta^{2}H$ (ANOVA, $F_{6,42} = 12.788$, $P<0.001$), $\log_{10}$ $\delta^{18}O$ ($F_{6,42} = 16.815$, $P<0.001$), $\delta^{13}C$ ($F_{6,63} = 13.205$, $P<0.001$) and $\delta^{15}N$ ($F_{6,63} = 16.613$, $P<0.001$).

$\delta^{2}H$ and $\delta^{18}O$

Haplotype group 1, representing *S. c. blythi* (sensu Olsson *et al.* 2013), differs significantly from virtually all other haplotype groups (*post hoc* Tukeys HSD, all $p<0.05$, Table 2a) – except haplotype 3, representing *S. c. margelanica* (sensu Olsson *et al.* 2013). Haplotype groups 2a, representing *S. c. halimodendri* (sensu Olsson *et al.* 2013) and 2b (*incertae sedis*, sensu Olsson *et al.,* 2013), had higher $\delta^{18}O$ values compared with haplotype group 3, ($p=0.009$ and $p=0.008$, respectively), but did not differ significantly in $\delta^{2}H$. There were no other statistically significant differences among haplotype groups (Table 2a).

$\delta^{13}C$ and $\delta^{15}N$

Haplotype group 1 had significantly lower $\delta^{13}C$ values compared with haplotype groups 2a, 2b and 5, the latter representing *S. c. curruca* (Table 2b). Differences between the remainder of the haplotype groups were not significant.

$\delta^{15}N$ values for haplotypes 2a and 2b were similar and were significantly higher compared with haplotypes 1, 3 and 5 (Table 2b, Fig. 3). Differences between the remainder of the haplotype groups were not significant (Table 2b, Fig. 3).

Discussion

Stable isotope ratios from lesser whitethroat feathers grown on the breeding grounds varied by haplotype group – differences were particularly strong between the allegedly intergrading *S. c. blythi* and *S. c. halimodendri.* This may be an indication that gene flow between these taxa is low or absent. Below we explore our findings, consider their shortcomings and consider their implications for understanding relationships among lesser whitethroat taxa and for using isotopes in other studies investigating links between phylogeny and ecology.

General caveats and limitations of stable isotope analysis

While the interpretation of isotope values is complex, here we generally assume that $\delta^{2}H$ and $\delta^{18}O$ are large-scale indicators, reflecting geographic origin based on spatial variation of precipitation isotopes (Bowen, *et al*. 2005). Thus samples from the same area should show similar stable isotope values, although local phenomena can also affect the values. For example, spatio-temporal differences in amounts of precipitation may result in different signatures, and evapotranspiration can increase $\delta^{18}O$ values in leaf tissue (Barbour 2007). Conversely, $\delta^{13}C$ and $\delta^{15}N$ reflect finer scale differences in habitat/diet choice, and vary as a function of changes in photosynthetic pathways and trophic enrichment (Inger and Bearhop 2008). These may thus vary between species living sympatrically, depending on factors pertaining to niche differentiation, such as microhabitat or food choice. $\delta^{15}N$ is primarily influenced by the trophic level of a species, but local-scale changes in $\delta^{15}N$ of plants may arise due to differing agricultural regimes or pollution close to urban areas. $\delta^{13}C$ is mainly influenced by the primary producers at the beginning of the food chain, with a species living in xeric habitat expected to be mostly influenced by food chains starting with C4 plants, but if their predominant prey comes from food chains starting with C3 plants, they would still show low $\delta^{13}C$ levels. Baseline values in plants may also vary greatly on a local scale due to e.g. fertilization or nitrification (West *et al*., 2010).

It is also important to bear in mind that lack of significant isotopic differences does not automatically equate to ecological similarity. Isotopic signatures in consumer tissues are a combination of a number of naturally occurring gradients and therefore it is possible for animals to occupy different habitats but have similar isotope values. One such potential problem with using isotope values is the inability to differentiate between isotopic gradients occurring as a function of continental-scale differences in rainfall patterns, and those that occur as a function of altitudinal gradients (Bowen, *et al*. 2005, Hobson, *et al*. 2004). In other words, it is possible that birds occupying different habitats may share similar isotopic niche signatures. The lack of differentiation between *S. c. halimodendri* and *S. c. althaea*, which

are partly sympatric but are altitudinally segregated (plains and mountains, respectively), may be an example of this.

It is clear therefore that isotopes are not wholly effective at delineating fine-scale habitat differences. Nevertheless, they are valuable in the context of the present study since they enable us to test for broad-scale ecological differences and similarities and particularly to determine whether morphological intermediate individuals exhibit intermediate ecologies.

Stable isotope differences between the* blythi *and* halimodendri *haplotype groups

Sylvia c. blythi (sensu Olsson *et al.,* 2013) is thought to primarily inhabit scrub and glades in the Siberian boreal forest region (Dement'ev and Gladkov, 1968; Shirihai *et al*., 2001). Our sample of this haplotype group differs significantly in δ^2H and δ^{18}O values from all other haplotype groups studied here, except from haplotype group 3 (representing *margelanica*, sensu Olsson *et al.,* 2013), further explored below. The significant differences in δ^2H and δ^{18}O values between *blythi* and *halimodendri*, reflecting overall habitat characteristics influenced by amount of rainfall, primarily corroborate the already known broad-scale differences in geographic location between these taxa. The most intriguing observation is that, despite their *halimodendri*-like plumage, all of our *blythi* haplotype samples show isotope signatures consistent with an origin in the mesic boreal forest region. This runs counter to the expectations of morphologically intermediate samples originating from the area between the semi-desert and the boreal forest. In such a scenario isotope signatures would have been expected to differ from typical *blythi* as well as *halimodendri*, by showing intermediate values of δ^2H and δ^{18}O.

Significant differences in δ^{13}C indicate that *blythi* and *halimodendri* occupy different habitats, may feed on prey that are part of different food chains (based on C3 plants or C4 and CAM plants, respectively), or both. The δ^{13}C values are significantly lower for *blythi* compared to

halimodendri, which occurs mainly in low altitude xeric habitats (Dement'ev and Gladkov, 1968, Olsson *et al.,* 2013, Shirihai *et al*., 2001). A possible explanation for the low $\delta^{13}C$ values in *blythi* is that the samples came from a habitat with food chains including a higher proportion of C3 plants, with the opposite effect expected in habitats dominated by C4 and CAM plants (Still *et al.* 2003). These differences corroborate the assumption that *blythi* is primarily a taxon of scrub and woodland (i.e. mesic habitats) and *halimodendri* is typically found in desert or xeric habitats.

Differences in $\delta^{15}N$ values are statistically significant between *blythi* and *halimodendri*, suggesting that these closely related taxa utilise different food sources. $\delta^{15}N$ values show a stepwise enrichment by a factor of 2.5–3 between subsequent trophic levels (Caut *et al*. 2009), indicating that *halimodendri* may on average feed at one or two trophic levels higher than *blythi*. It is possible that primary consumer prey items, e.g. lepidopteran larvae, are more abundant in mesic habitats. However, although differences in $\delta^{15}N$ values are usually taken to indicate differences in trophic enrichment, the interpretation is complicated by $\delta^{15}N$ values also being generally lower in mesic than in xeric habitats (Kelly 2000). Furthermore, presence of grazing livestock may contribute to increased levels of $\delta^{15}N$ (Kerley and Jarvis, 1996). It is possible that there are more grazing animals in the arid areas of Central Asia than in the temperate forest region, contributing to this difference. Consequently, the predictive value of differences in $\delta^{15}N$ increases in cases when the samples come from the same area, particularly in cases of possible niche overlap. In the case of *blythi* and *halimodendri* there is a possibility that the differences are influenced by other factors than different feeding habits.

Even if the differences in general between *blythi* and *halimodendri* need to be interpreted with caution (see *General caveats and limitations of stable isotope analysis* above), the origin of the differences is in this context less important than the fact that they indicate

significant ecological division between *blythi* and *halimodendri* on the breeding grounds, i.e. that they occupy different niches. The correlation between haplotype and geographic distribution reported by Olsson et al. (2013) also indicate that they breed in different areas. Furthermore, the niche utilization does not seem to be correlated to the external morphology in *blythi*, as our entire sample of this taxon was morphologically more similar to the desert forms than to *curruca*-like typical *blythi*. Hypothetically, *halimodendri*-like plumage could be an indication that they originated from a population under selection for similar exterior morphology as *halimodendri*, i.e. one living in *halimodendri*-like habitat. However, our isotope data clearly indicate that all samples with a *blythi* haplotype originated from boreal forest habitat. Furthermore, all individuals collected by Olsson *et al* (2013) on the breeding grounds in the temperate forest belt belonged to the distinct *blythi* haplotype group, and all individuals collected during the summer months on the breeding grounds in Central Asian arid semi-desert belonged to the *halimodendri* haplotype.

Implications of isotopic niche differentiations among other taxa

The reason for the lack of differentiation in δ^2H and δ^{18}O values between *blythi* and *margelanica* is not clear. Possibly *margelanica* occurs in areas with a higher amount of precipitation than the other southerly populations, resulting in an isotopic signature more similar to those further north and highlighting the shortcomings of using geographic range alone as a proxy for habitat selection. Differences in δ^{18}O values between *margelanica* and *halimodendri* are significant and consistent with the δ^2H results, the latter appearing to inhabit the most xeric habitat.

Differences in δ^{13}C values are significantly lower for *blythi* compared to *minula*, which occurs mainly in low altitude xeric habitats (Olsson *et al.,* 2013). Differences in δ^{13}C values between *blythi* and the two high altitude haplotype groups *althaea* and *margelanica* are not statistically significant, but the values indicate that the habitats of *althaea* and *margelanica*

may be intermediate between that of *blythi,* and that of *minula* and *halimodendri* in this respect.

δ^{15}N values differ significantly between the three southern forms. *halimodendri* on the one hand and *margelanica* and *minula* on the other, where habitat differences are less obvious, and the reason for this is unclear. In contrast, there are no significant differences in δ^{15}N values between the northern *blythi* and the southern *margelanica* and *minula* in spite of obvious habitat differences (Olsson *et al.,* 2013; Shirihai *et al.,* 2001). Between *blythi* and *althaea* differences in δ^{15}N values are nearly statistically significant (p=0.069), although the implications of this are unclear.

A possible conclusion from δ^{13}C and δ^{15}N values may be that *halimodendri* feeds at a higher trophic level or in areas with longer food chain lengths compared with *blythi, margelanica* and *minula*, respectively.

Apart from the cases highlighted above, there were no other consistent patterns in terms of differences in stable isotope ratios between haplotype groups. Interestingly, there are no statistically significant differences in stable isotope ratios between *halimodendri* and *althaea*, which breed sympatrically, but are segregated by altitude. It is well known that these birds are morphologically divergent from each other, and they are considered separate species by most authors, based on evidence of reproductive isolation (Korelov, 1972; Loskot, 2001; Shirihai *et al*., 2001; Stepanyan, 1983). More subtle differences in environmental conditions such as the amount of precipitation and composition of plant communities may exist but are not large enough to leave an imprint on the isotopic signatures.

Loskot (2005) and Williamson (1976) suggested that *minula* and *margelanica* intergrade in the Qaidam depression, but we have no isotope data from this area. Given their lack of

409 differentiation in isotopic signature from other areas, indicating similar ecological
410 requirements, populations in this area would make an interesting case study regarding
411 amount of niche overlap, heterospecific interaction and selection against hybrids.
412
413 ***Concluding remarks***
414 In this paper we illustrate how the analysis of isotopic niche can be used in tandem with
415 phylogenetic information to explore links between ecological divergence and genetic
416 differentiation within a closely related group of birds, and how this method of reciprocal
417 illumination allows valuable insights into the speciation process.
418
419 Our data suggest complete correlation between isotope signature and haplotype between
420 *halimodendri* and *blythi*, clearly indicating that *halimodendri* occupies a different niche than
421 birds with *halimodendri*-like appearance but *blythi* haplotype. There are no indications of
422 intermediate, less habitat specific, individuals among our *halimodendri* and *blythi* samples,
423 and no haplotypes typical of one taxon were detected in individuals breeding in habitat more
424 characteristic of the other taxon. This finding casts some doubt on the generally accepted
425 assumption that morphologically intermediate Lesser Whitethroats originate from a
426 population of intergrades inhabiting the region where semi-desert grades into the boreal
427 forest. The strict differentiation into isotopic niches also by intermediate-looking individuals
428 and a lack of individuals with “misplaced” haplotypes speak against an extensive hybrid
429 zone, but does not reject it. There is a risk that we may have entirely missed a population
430 representing the hybrid zone. This could happen by chance as our sample is small, or if, for
431 example, different populations migrate along different routes or at different times. However,
432 these limitations apply to the design of this study, not the approach in general. With carefully
433 designed sampling that ensures samples of both taxa from the area of range overlap are
434 included, this method should provide information about habitat and food preferences of
435 different populations that could add valuable evidence for the determination of species limits.
436

Studies in a contact zone of both *blythi* and *halimodendri* and other taxon pairs, such as *minula* and *margelanica*, has the potential to shed light on the role of character displacement as a driver of morphological and ecological divergence in the early stages of secondary contact. Another outstanding question is whether the morphological and ecological divergence found between sympatric species is instead driven by adaptations in allopatry, and must already be in place before sympatry is possible. Future research on the interactions, habitat preferences and food choice of these taxa in the transition zone between their distribution ranges should provide important insights into the most crucial step of the speciation process, the build-up of sympatric diversity.

Acknowledgements

We would like to thank Andrew Grieve, Andrew Lassey, Peter Kennerley, Annika Forsten, Lars Svensson, Brydon Thomason and Tony & Helen Mainwood for help in the field and for helpful discussions. We are very grateful to Edward Gavrilov and Martin Stervander for providing samples. This work was funded by Jornvall Foundation (to P.A.), the Sound Approach (to P.A. and U.O.), the Chinese Academy of Sciences Visiting Professorship for Senior International Scientists (No. 2011T2S04, to P.A.) and a grant from the Natural Environment Research Council (for stable isotope analysis) and the Eric Hosking Charitable Trust (to SCV).

459 **Table 1.** Details of sampling locations, period of the annual cycle and year of Lesser Whitethroat taxa used in stable isotope analysis.

Allospecies	Site	Country	Status	Year sampled	Year feather grown	Haplotype	Latitude	Longitude	*n*
althea	Issyk Kol	Kyrgyzstan	On breeding grounds	2003	2002	4	42°25'N	76°70'E	1
althea	Turaigyr	Kazakhstan	On breeding grounds	2003	2002	4	43°29'N	78°38'E	2
althea	Zhetyzhol	Kazakhstan	On breeding grounds	2003	2002	4	43°01'N	76°03'E	1
blythi	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	1	24°16'N	52°52'E	2
blythi	Chokpak	Kazakhstan	Migrating	2002	2001	1	42°31'N	70°38'E	14
blythi	Balkash	Kazakhstan	Migrating	2002	2001	1	46°70'N	74°35'E	1
blythi	Ili River	Kazakhstan	Migrating	2002	2001	1	45°07'N	75°26'E	1
blythi	Sorbulak	Kazakhstan	Migrating	2002	2001	1	43°70'N	76°50'E	3
halimodendri	Al Wathba, Abu	United Arab Emirates	Wintering	2004	2003	2a	24°15'N	54°40'E	3
halimodendri	Al Wathba, Abu	United Arab Emirates	Wintering	2004	2003	2b	24°15'N	54°40'E	2
halimodendri	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	2a	24°16'N	52°52'E	6
halimodendri	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	2b	24°16'N	52°52'E	10
halimodendri	Ili River	Kazakhstan	On breeding grounds or migrating	2003	2003	2a	45°07'N	75°26'E	2
halimodendri	Sorbulak	Kazakhstan	On breeding grounds or migrating	2003	2003	2a	43°70'N	76°50'E	3
halimodendri	Sorbulak	Kazakhstan	On breeding grounds or migrating	2003	2003	2b	43°70'N	76°50'E	1
halimodendri	Hilf	Oman	Wintering	2003	2003	2a	20°66'N	58°90'E	1
halimodendri	Khatmat Milahah	Oman	Wintering	2003	2003	2a	24°95'N	56°35'E	1
halimodendri	Hilf	Oman	Wintering	2003	2003	2b	20°66'N	58°90'E	1
halimodendri	Khatmat Milahah	Oman	Wintering	2003	2003	2b	24°95'N	56°35'E	3
halimodendri	Xinjiang	China	September – Breeding grounds?	2004	2004	2b	42°09'N	89°02'E	1
margelanica	Qinghai	China	On breeding grounds	2003	2002	3	36°30'N	100°60'E	1
margelanica	Xinjiang	China	September – Breeding grounds?	2004	2004	3	42°09'N	89°02'E	4
minula	Xinjiang	China	On breeding grounds	2003	2002	5	40°05'N	81°04'E	9
minula	Xinjiang	China	September – Breeding grounds?	1998	1998	5	42°09'N	89°02'E	1

460

461 **Table 2.** Stable isotope signatures vary as a function of lesser whitethroat haplotype.
462 Results of *post hoc* Tukey HSD multiple comparisons for differences in: (a) δ^2H and (log)
463 δ^{18}O, primarily reflecting geographical differences and; (b) δ^{13}C and δ^{15}N primarily reflecting
464 small-scale differences in habitat occupancy and foraging behaviour. Mean differences are
465 presented alongside p-values – statistically significant differences at alpha level 0.05 are
466 highlighted in bold.

467

(a)	Haplotype				
	1	2a	2b	3	4
δ^2H					
2a	**-49.26, p<0.001**	-			
2b	**-49.79, p<0.001**	-0.53, p=1.000	-		
3	-25.07, p=0.058	24.21, p=0.240	24.74, p=0.181	-	
4	**-47.53, p<0.001**	1.72, p=1.000	2.26, p=1.000	-22.48, p=0.379	-
5	**-40.34, p<0.001**	8.91, p=0.930	9.44, p=0.890	-15.30, p=0.580	7.18, p=0.980
δ^{18}O					
2a	**-0.25, p<0.001**	-			
2b	**-0.24, p<0.001**	0.01, p=1.000	-		
3	-0.08, p=0.145	**0.16, p=0.006**	**0.16, p=0.005**	-	
4	**-0.18, p<0.001**	0.06, p=0.762	0.06, p=0.796	-0.09, p=0.253	-
5	**-0.14, p<0.001**	0.10, p=0.081	0.10, p=0.077	-0.06, p=0.619	0.04, p=0.897

468

(b)	Haplotype				
	1	2a	2b	3	4
δ^{13}C					
2a	**-4.81, p<0.001**	-			
2b	**-4.06, p<0.001**	0.75, p=0.855	-		
3	-2.23, p=0.114	2.58, p=0.070	1.83, p=0.303	-	
4	-2.24, p=0.177	2.56, p=0.121	1.81, p=0.412	-0.02, p=1.000	-
5	**-2.79, p=0.001**	2.01, p=0.085	1.27, p=0.435	-0.56, p=0.991	-0.54, p=0.995
δ^{15}N					
2a	**-4.11, p<0.001**	-			
2b	**-4.51, p<0.001**	-0.40, p=0.987	-		
3	-0.44, p=0.995	**3.67, p=0.001**	**4.07, p<0.001**	-	
4	-2.55, p=0.069	1.56, p=0.586	1.96, p=0.285	-2.11, p=0.417	-
5	-1.41, p=0.253	**2.71, p=0.004**	**3.11, p<0.001**	-0.97, p=0.895	1.14, p=0.853

469
470

Figure 1. Breeding range of five main Lesser Whitethroat taxa as described by Shirihai *et al*. (2001). A sixth taxon, *Sylvia c. blythi* (sensu Olsson *et al.,* 2013) is thought to primarily inhabit scrub and glades in the Siberian boreal forest region.

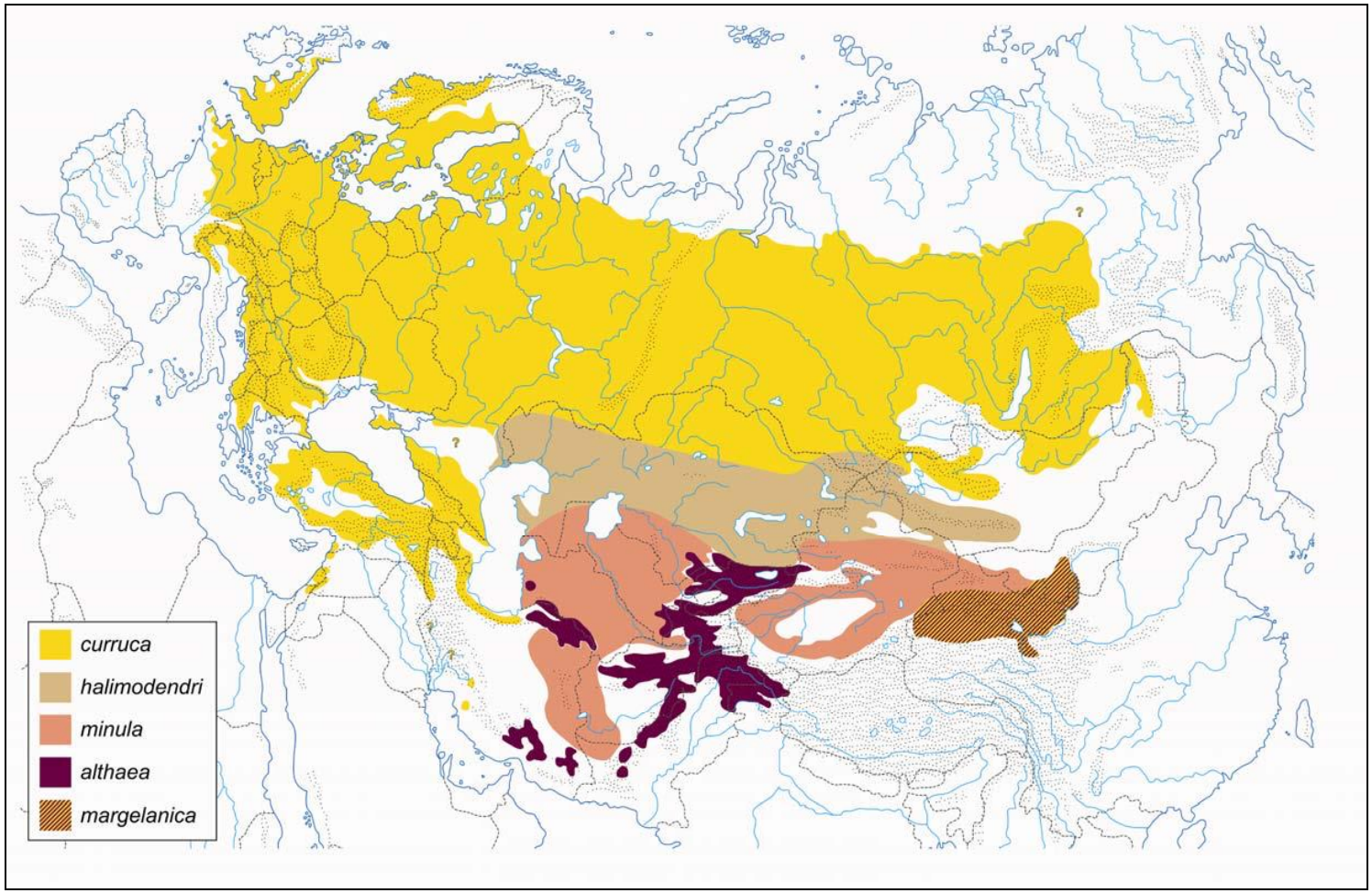


Figure 2. Phylogeny of the Asian Lesser Whitethroat taxa after Olsson *et al*. (2013). The unnamed sister taxon of *halimodendri* is here treated as synonymous with *halimodendri*.

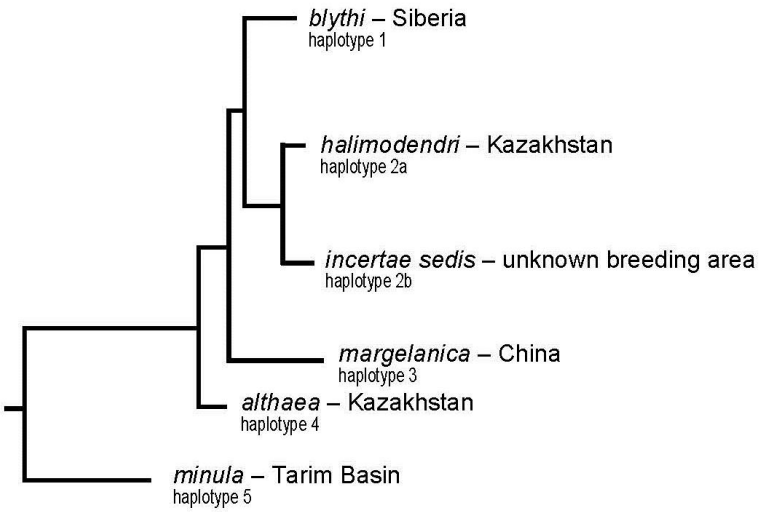
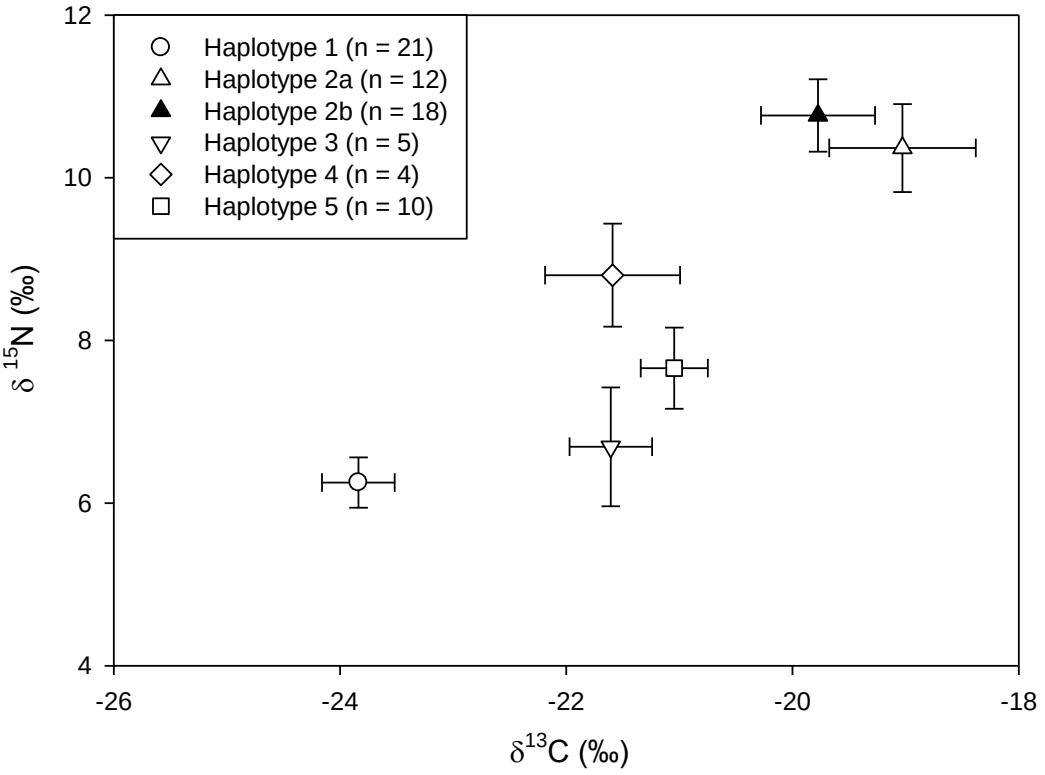
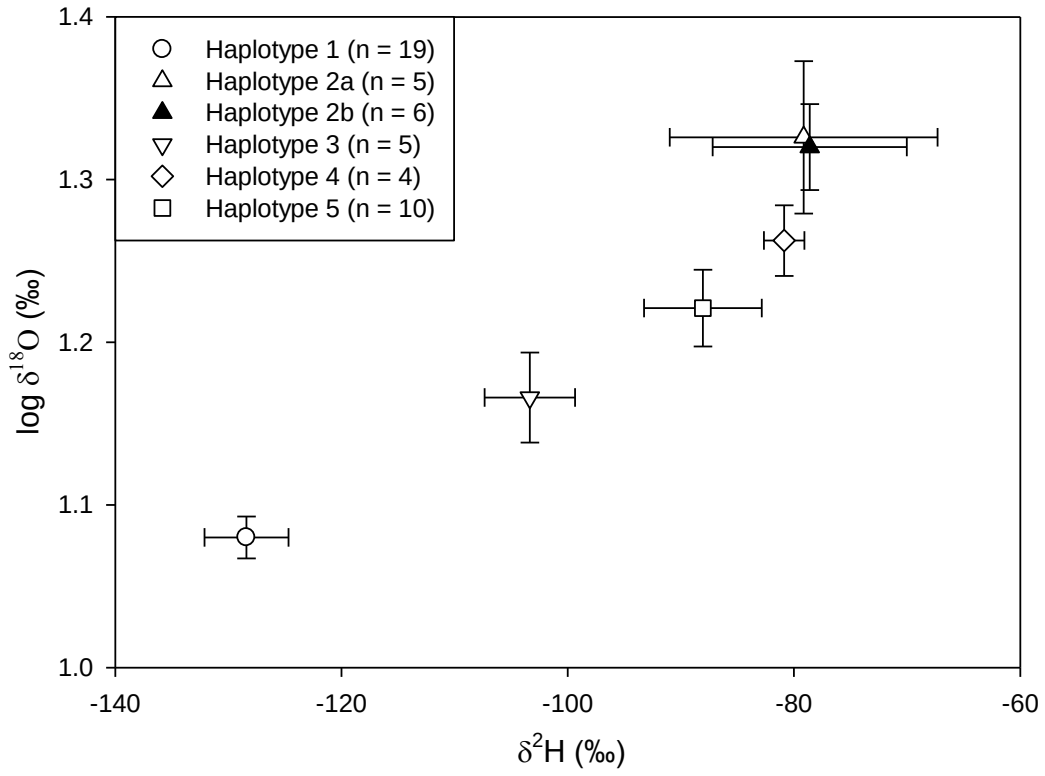


Figure 3 Stable isotopes of hydrogen, carbon, nitrogen and oxygen vary as function of six haplotype groups in the lesser whitethroat complex. Haplotype 1 equals *blythi*, haplotypes 2a and 2b equal *halimodendri*, haplotype 3 equals *margelanica*, haplotype 4 equals *althaea* and haplotype 5 equals *minula*. Values are means ±1SE. In both contrasts, significant differences are found between *blythi* on the lower left and *halimodendri* on the upper right. The other taxa show intermediate stable isotope ratios.


Haplotype 1 (n = 21)
Haplotype 2a (n = 12)
Haplotype 2b (n = 18)
Haplotype 3 (n = 5)
Haplotype 4 (n = 4)
Haplotype 5 (n = 10)
12
10
8
6
4
-26
-24
-22
-20
-18
δ 15N (‰)
δ13C (‰)


Haplotype 1 (n = 19)
Haplotype 2a (n = 5)
Haplotype 2b (n = 6)
Haplotype 3 (n = 5)
Haplotype 4 (n = 4)
Haplotype 5 (n = 10)
1.4
1.3
1.2
1.1
1.0
-140
-120
-100
-80
-60
log δ18O (‰)
δ2H (‰)

REFERENCES

Barbour, M. G. 2007. Stable oxygen isotope composition of plant tissue: a review. Functional Plant Biology, 34, 83**–**94.

Bearhop, S., Adams, C. E., Waldron, S., Fuller, R. A. and Macleod, H. 2004. Determining trophic niche width: a novel approach using stable isotope analysis. - Journal of Animal Ecology 73: 1007-1012.

Bearhop, S., Furness, R.W., Hilton, G.M., Votier, S.C. & Waldron, S. 2003. A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. Functional Ecology 17: 270–275

Boecklen, W.J., Yarnes, C.T., Cook, B.A. & James, A.C. 2011. On the use of stable isotopes in trophic ecology. Annu. Rev. Ecol. Evol. Syst. 42: 411**–**440.

Bowen, G. J., Wassenaar, L. I. and Hobson, K. A. 2005. Global application of stable hydrogen and oxygen isotopes to wildlife forensics. - Oecologia 143: 337-348.

Caut, S., Angulo, E., Courchamp, F. 2009. Variation in discrimination factors (δ^{15}N and δ^{13}C): the effect of diet isotopic values and applications for diet reconstruction. J Appl Ecol 46:443–453

Charmantier, A., Blondel, J., Perret, P., Harmelin-Vivien, M. 2014. Tracing site specific signatures along a Blue Tit *Cyanistes caeruleus* food chain. Ibis 156: 165-175.

Chesson, P. 2000. Mechanisms of maintenance of species diversity. *Annual Review of Ecology and Systematics* **31,** 343–366.

Cramp, S. 1992. The Birds of the Western Palearctic Vol. 6. - Oxford University Press.

del Hoyo, J. E., A. & Christie D. 2006. Handbook of the Birds of the World. Volume 11: Old World Flycatchers to Old World Warblers. - Lynx Edicions.

Dement'ev, G.P., Gladkov. N.A. eds. 1968. Birds of the Soviet union, vol. 6. Jerusalem: Israel Program for Scientific Translations..

DeNiro, M. J., Epstein, S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta, 45, 341–351.

Endler, J.A. 1973. Gene flow and population differentiation. Science, 179,243-250.

Endler, J.A. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, New Jersey.

Goldberg, E.E., and Lande, R. 2006. Ecological and reproductive character displacement on an environmental gradient. *Evolution* **60,** 1344–1357.

Hasegawa, M., Kishino, H. and Yano, T. A. 1985. Dating of the Human Ape Splitting by a Molecular Clock of Mitochondrial-DNA. - Journal of Molecular Evolution 22: 160-174.

Hobson, K. A., Bowen, G. J., Wassenaar, L. I., Ferrand, Y. and Lormee, H. 2004. Using stable hydrogen and oxygen isotope measurements of feathers to infer geographical origins of migrating European birds. - Oecologia 141: 477-488.

Inger, R. and Bearhop, S. 2008. Applications of stable isotope analyses to avian ecology. - Ibis 150: 447-461.

Kelly, J. F. 2000. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. - Canadian Journal of Zoology-Revue Canadienne De Zoologie 78: 1-27.

Kerley, S.J., Jarvis, S.C. 1996. Preliminary studies of the impact of excreted N on cycling and uptake of N in pasture systems using natural abundance stable isotopic discrimination. Plant Soil. 178: 287-294.

Korelov, M.N., 1972. Genus Sylvia (in Russian). In: Korelov, M.N., Kovshar, A.F. (Eds.), Birds of Kazakhstan (Ptitsy Kazakhstana), vol. 4. Alma-Ata, pp. 153–205.

Loskot, V.M., 2001. Taxonomic revision of the Hume's Whitethroat Sylvia althaea Hume, 1878. Avian. Ecol. Behav. 6, 41–42.

Loskot, V. M. 2005. Morphological variation and taxonomic revision of five south-eastern subspecies of Lesser Whitethroat Sylvia curruca (L.) (Aves: Sylviidae). - Zool. Med. Leiden 79-3 (15).

Mayr, E. C., G.W. 1986. Check-list of Birds of the World. - Harvard University Press.

Mayr, E. & Diamond, J. M. 2001. *The Birds of Northern Melanesia: Speciation, Ecology, and*

Biogeography. (Oxford University Press).
Mizutani, H., Fukuda, M., Kabaya, Y., Wada, E. 1990. Carbon isotope ratio of feathers reveals feeding behavior of cormorants. Auk 107:400-403
Newsome, S. D., del Rio, C. M., Bearhop, S. and Phillips, D. L. 2007. A niche for isotopic ecology. - Frontiers in Ecology and the Environment 5: 429-436.
Newton, J. (2010) Continuous-flow isotope ratio mass spectrometry with an elemental analyzer: oxidative approaches for carbon and nitrogen isotopes. In: Beauchamin, D. and Matthews, D.E. (eds.) Elemental and Isotope Ratio Mass Spectrometry. Series: The Encyclopedia of Mass Spectrometry, 5. Elsevier, pp. 759-764
Olsson, U., Alström, P., Leader, P.J., Carey, G.J., Svensson, L. 2013. New insights into the intricate taxonomy and phylogeny of the Sylvia curruca complex. Mol. Phyl. Evol. 67: 72-85.
Peterson, B.J., Fry, B. 1987. Stable isotopes in ecosystem studies. Annu Rev Ecol Syst 18:293-320
Portenko, L. A. 1960. The birds of the USSR. Part IV. - USSR Academy of Sciences 69: 1-413.
Posada, D., 2008a. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Posada, D, 2008b. jModeltest. version 0.1.1. Available at https://code.google.com/p/jmodeltest2.
Post, D.M. 2002. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology, 83(3). 703-718.
Price, T. 2008. *Speciation in Birds*. (Roberts and Co).
Price, T. D. & Bouvier, M. M. 2002. The evolution of F1 postzygotic incompatibilities in birds. *Evolution* **56,** 2083–2089.
Price, T. D., D. M. Hooper, C. D. Buchanan, U. S. Johansson, D. T. Tietze, P. Alström, U. Olsson, M. Ghosh-Harihar, F. Ishtiaq, S. K. Gupta, J. Martens, B. Harr, P. Singh and D. Mohan. (2014) Niche filling slows the diversification of Himalayan songbirds. Nature 509: 222–225
Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. - Bioinformatics 19: 1572-1574.
Schell, D.M., Saupe, S.M., Haubenstock, N. 1989. Bowhead whale (Balaena mysticetus) growth and feeding as estimated by **d**13C techniques. Mar Biol 103: 433-443
Shirihai, H., Gargallo, G., Helbig, A., Harris, A., Cottridge, D. and Roselaar, D. 2001. Sylvia warblers: Identification, taxonomy and phylogeny of the genus Sylvia. - Christopher Helm, A & C Black.
Stepanyan, L.S., 1983. Superspecies and sibling species in avifauna of the USSR (Nadvidy i vidy-dvoyniki v avifaune SSSR). Moscow, pp. 1–194 (in Russian).
Still, C.J., Berry, J.A., Collatz, G.J. and DeFries, R.S. 2003. Global distribution of C-3 and C-4 vegetation: Carbon cycle implications. - Global Biogeochemical Cycles 17 (1), 1006, doi:10.1029/2001GB001807.
Svensson, L. 1992. Identification guide to European passerines. - British Trust for Ornithology.
Tieszen, L.L., Boutton, T.W., Tesdahl, K.G., Slade, N.A. 1983. Fractionation and turnover of stable carbon isotopes in animal tissues: implications for **d**13C analysis of diet. Oecologia 57:32-37
Vanderklift, M.A. & Ponsard, S. 2003. Sources of variation in consumer-diet delta(15)N enrichment: a meta-analysis. Oecologia 136: 169**–**182.
Vaurie, C. 1959. The birds of the Palearctic fauna: A systematic reference, order Passeriformes. - H.F. & G. Witherby
Wassenaar, L.I. and Hobson, K.A. 2000.Stable carbon and hydrogen isotope ratios reveal breeding origins of red-winged blackbirds. Ecol. Appl., 10, 911–916.
Wassenaar, L.I. and Hobson, K.A. 2003. Comparative equilibration and online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. Isotopes in Environmental and Health Studies 39, (3): 211–217

Weir, J.T., Price, T.D. 2011. Limits to speciation Inferred from times to secondary sympatry and ages of hybridizing species along a latitudinal gradient. *Am. Nat.* **177,** 462–469.

West, J.B., Bowen, G.J., Cerling, T.E., Ehleringer, J.R. 2006. Stable isotopes as one of nature's ecological recorders. Trends Ecol Evol 21:408–414

West, J.B., Bowen, G.J., Dawson, T.E., Tu, K.P. 2010. Isoscapes: understanding movement, pattern and process on earth through isotope mapping. Springer, New York

Williamson, K. 1976. Identification for Ringers. The genus Sylvia. BTO Field Guide 9. - British Trust for Ornithology.

Wright, S. 1943. Isolation by distance. Genetics 28:114–138.

Yang, Z. H. 1994. Maximum-Likelihood Phylogenetic Estimation from DNA-Sequences with Variable Rates over Sites - Approximate Methods. - Journal of Molecular Evolution 39: 306-314.