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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)

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

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26

27

28 **Abstract**

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

49

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

52

53

54

55 Introduction

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

64

65 The Lesser Whitethroat complex (*Sylvia curruca sensu lato*, Sylviidae, Passeriformes, Aves)
66 is a group of morphologically similar insectivorous warblers, breeding across almost the
67 entire Palearctic, from Western Europe to east Siberia, and southwards through
68 northwestern China and Central Asia to south-western Iran (Cramp 1992, del Hoyo 2006,
69 Mayr 1986, Olsson *et al.* 2013, Shirihai *et al.* 2001, Vaurie 1959) (Fig 1). The subtle
70 morphological variation between the different taxa in this complex has long obscured the
71 taxonomy, but Olsson *et al.* (2013) proposed, based on analyses of mitochondrial DNA, that
72 it consists of six well supported clades (Fig. 2). Four of these clades, representing *S. c.*
73 *althaea*, *S. c. blythi*, *S. c. halimodendri* and *S. c. margelanica*, occupy more or less
74 parapatric ranges in Central Asia, which share a most recent common ancestor 1.95 ± 0.55
75 million years ago (Olsson *et al.*, 2013). Consequently, these clades seem to be mainly below
76 the critical two million year level of divergence, offering one possible explanation as to why
77 they do not occur in sympatry. Several authors point out that there seems to be a
78 morphological cline between *S. c. blythi* of the Siberian boreal forests and *S. c. halimodendri*
79 of Central Asian semi-desert (Loskot, 2005; Shirihai *et al.*, 2001; Vaurie, 1959). A
80 morphological cline could arise if reproductive barriers between two previously separated
81 populations are incomplete or break down after they come into secondary contact (Endler,
82 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.

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

114
115 Unfortunately, diagnosis based on mitochondrial DNA data is almost equally unhelpful when
116 it comes to diagnosing morphologically intermediate individuals. Although mitochondrial
117 haplotype will unambiguously assign an individual to one of the clades identified by Olsson
118 *et al.* (2013), it will not reveal whether an individual that is morphologically intermediate is of
119 hybrid origin or just represent an extreme end of a within-taxon morphological variation.
120 Moreover, Olsson et al. (2013) found no fixed differences between these taxa in nuclear
121 markers. The most likely reason for this is that the time since these lineages diverged is too
122 short for fixed differences to occur in these markers. For these reasons, we sought ways to
123 indirectly collect information pertaining to the ecological requirements and relationships of
124 these two taxa.

125
126 The analysis of stable isotope ratios has emerged as a powerful tool for ecological study in
127 recent decades (e.g. Bearhop *et al.*, 2004; Boecklen *et al.*, 2011; Bowen *et al.* 2005;
128 Charmantier *et al.*, 2014; Inger & Bearhop 2008; Newsome *et al.*, 2007; Post, 2002; West *et*
129 *al.*, 2006), and, in the context of the present study, may be helpful in resolving ecological
130 differences among the closely related members of the lesser whitethroat complex in general
131 and *blythi*/*halimodendri* in particular. This approach relies on the fact that naturally occurring
132 gradients in stable isotopes are reflected in consumer tissues in a predictable manner. Some
133 keratinous tissues like hair, feather or nail are metabolically inert following synthesis and so
134 maintain an isotopic record reflecting the location where the tissue was synthesized (Schell
135 *et al.* 1989; Mizutani *et al.* 1990). Moreover, in the case of feathers they provide data
136 covering the period over which they are grown (weeks to months; Bearhop et al. 2003). This
137 time-integrated information on organic carbon sources for heterotrophs and information on
138 habitat of origin may be more informative than observations of habitat unless the latter are

139 conducting over extended periods.

140

141 Here we measure isotopes of hydrogen ($\delta^2\text{H}$), carbon ($\delta^{13}\text{C}$), nitrogen ($\delta^{15}\text{N}$) and oxygen
142 ($\delta^{18}\text{O}$) in Lesser Whitethroat feathers grown during the breeding to provide information on
143 food choice and habitat use. The ratio of heavy to light hydrogen $^1\text{H}:^2\text{H}$ (expressed as $\delta^2\text{H}$)
144 and oxygen isotopes $^{16}\text{O}:^{18}\text{O}$ (expressed as $\delta^{18}\text{O}$) vary largely because of isotopic
145 fractionation during the phase change for vapour to liquid or solid associated with
146 precipitation, with the proportion of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ decreasing on a continuous scale with
147 increasing latitude, with distance from the sea and with increasing altitude (Bowen, *et al.*
148 2005). We here use $\delta^2\text{H}$ and $\delta^{18}\text{O}$ primarily as broad-scale markers of distribution within the
149 Lesser Whitethroat complex, as well as to test for altitudinal differences among clades. The
150 ratio of heavy to light carbon isotopes $^{12}\text{C}:^{13}\text{C}$ (expressed as $\delta^{13}\text{C}$) in primary producers
151 (and therefore in upper trophic levels too) varies on a discrete scale as a function of different
152 photosynthetic pathways – C3 plants having lower ^{13}C values compared with plants utilising
153 C4 photosynthetic pathways, with plants utilising crassulacean acid metabolism (CAM) being
154 somewhat intermediate but with ^{13}C values usually most similar to C3 plants (Peterson and
155 Fry 1987; Tieszen *et al.* 1983). The ratio of $^{14}\text{N}:^{15}\text{N}$ (expressed as $\delta^{15}\text{N}$) increases with
156 each trophic level (DeNiro and Epstein 1981), and, although interpretations are not always
157 straightforward (Vanderklift and Ponsard, 2003), may contribute evidence of possible
158 differences in food choice.

159

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

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

176

177 **Methodology**

178

179 ***Taxonomic names and sampling***

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

221 IRMS on the same instrumentation, but using the Costech HTG-02 reactor (see Newton
222 2010 for description). Isotope ratios are reported as δ -values and expressed as ‰ according
223 to the equation $\delta X = [R_{\text{sample}}/R_{\text{standard}} - 1] \times 1000$, where X is ^2H , ^{13}C , ^{15}N or ^{18}O and R is the
224 corresponding ratio $^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ or $^{18}\text{O}/^{16}\text{O}$ and R_{standard} is the ratio of the
225 international references for each element. Hydrogen isotope analysis of feather samples is
226 not straightforward since around a fifth of the hydrogen in keratin can exchange readily with
227 ambient water vapour. We used the comparative equilibration procedure of Wassenaar and
228 Hobson (2000, 2003) and the CFS and BWB-II standards reported there, to correct for non-
229 indigenous hydrogen.

230

231 **Statistical analysis**

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

239

240 **Results**

241 Overall there were significant isotopic differences among the six lesser whitethroat
242 haplotypes (Fig. 3, Table 2; MANOVA, Wilk's $\lambda = F_{24, 137.3} = 4.59$, $P < 0.001$, Figure 3) and
243 univariate analysis revealed differences among $\delta^2\text{H}$ (ANOVA, $F_{6,42} = 12.788$, $P < 0.001$), \log_{10}
244 $\delta^{18}\text{O}$ ($F_{6,42} = 16.815$, $P < 0.001$), $\delta^{13}\text{C}$ ($F_{6,63} = 13.205$, $P < 0.001$) and $\delta^{15}\text{N}$ ($F_{6,63} = 16.613$,
245 $P < 0.001$).

246

247 **$\delta^2\text{H}$ and $\delta^{18}\text{O}$**

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

255

256 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

257 Haplotype group 1 had significantly lower $\delta^{13}\text{C}$ values compared with haplotype groups 2a,
258 2b and 5, the latter representing *S. c. curruca* (Table 2b). Differences between the remainder
259 of the haplotype groups were not significant.

260

261 $\delta^{15}\text{N}$ values for haplotypes 2a and 2b were similar and were significantly higher compared
262 with haplotypes 1, 3 and 5 (Table 2b, Fig. 3). Differences between the remainder of the
263 haplotype groups were not significant (Table 2b, Fig. 3).

264

265 Discussion

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

272

273 **General caveats and limitations of stable isotope analysis**

274 While the interpretation of isotope values is complex, here we generally assume that $\delta^2\text{H}$
275 and $\delta^{18}\text{O}$ are large-scale indicators, reflecting geographic origin based on spatial variation of
276 precipitation isotopes (Bowen, *et al.* 2005). Thus samples from the same area should show
277 similar stable isotope values, although local phenomena can also affect the values. For
278 example, spatio-temporal differences in amounts of precipitation may result in different
279 signatures, and evapotranspiration can increase $\delta^{18}\text{O}$ values in leaf tissue (Barbour 2007).
280 Conversely, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ reflect finer scale differences in habitat/diet choice, and vary as a
281 function of changes in photosynthetic pathways and trophic enrichment (Inger and Bearhop
282 2008). These may thus vary between species living sympatrically, depending on factors
283 pertaining to niche differentiation, such as microhabitat or food choice. $\delta^{15}\text{N}$ is primarily
284 influenced by the trophic level of a species, but local-scale changes in $\delta^{15}\text{N}$ of plants may
285 arise due to differing agricultural regimes or pollution close to urban areas. $\delta^{13}\text{C}$ is mainly
286 influenced by the primary producers at the beginning of the food chain, with a species living
287 in xeric habitat expected to be mostly influenced by food chains starting with C4 plants, but if
288 their predominant prey comes from food chains starting with C3 plants, they would still show
289 low $\delta^{13}\text{C}$ levels. Baseline values in plants may also vary greatly on a local scale due to e.g.
290 fertilization or nitrification (West *et al.*, 2010).

291

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

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

303

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

308

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

310 *Sylvia c. blythi* (sensu Olsson *et al.*, 2013) is thought to primarily inhabit scrub and glades in
311 the Siberian boreal forest region (Dement'ev and Gladkov, 1968; Shirihai *et al.*, 2001). Our
312 sample of this haplotype group differs significantly in $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values from all other
313 haplotype groups studied here, except from haplotype group 3 (representing *margelanica*,
314 sensu Olsson *et al.*, 2013), further explored below. The significant differences in $\delta^2\text{H}$ and
315 $\delta^{18}\text{O}$ values between *blythi* and *halimodendri*, reflecting overall habitat characteristics
316 influenced by amount of rainfall, primarily corroborate the already known broad-scale
317 differences in geographic location between these taxa. The most intriguing observation is
318 that, despite their *halimodendri*-like plumage, all of our *blythi* haplotype samples show
319 isotope signatures consistent with an origin in the mesic boreal forest region. This runs
320 counter to the expectations of morphologically intermediate samples originating from the
321 area between the semi-desert and the boreal forest. In such a scenario isotope signatures
322 would have been expected to differ from typical *blythi* as well as *halimodendri*, by showing
323 intermediate values of $\delta^2\text{H}$ and $\delta^{18}\text{O}$.

324

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

328 *halimodendri*, which occurs mainly in low altitude xeric habitats (Dement'ev and Gladkov,
329 1968, Olsson *et al.*, 2013, Shirihai *et al.*, 2001). A possible explanation for the low $\delta^{13}\text{C}$
330 values in *blythi* is that the samples came from a habitat with food chains including a higher
331 proportion of C3 plants, with the opposite effect expected in habitats dominated by C4 and
332 CAM plants (Still *et al.* 2003). These differences corroborate the assumption that *blythi* is
333 primarily a taxon of scrub and woodland (i.e. mesic habitats) and *halimodendri* is typically
334 found in desert or xeric habitats.

335

336 Differences in $\delta^{15}\text{N}$ values are statistically significant between *blythi* and *halimodendri*,
337 suggesting that these closely related taxa utilise different food sources. $\delta^{15}\text{N}$ values show a
338 stepwise enrichment by a factor of 2.5–3 between subsequent trophic levels (Caut *et al.*
339 2009), indicating that *halimodendri* may on average feed at one or two trophic levels higher
340 than *blythi*. It is possible that primary consumer prey items, e.g. lepidopteran larvae, are
341 more abundant in mesic habitats. However, although differences in $\delta^{15}\text{N}$ values are usually
342 taken to indicate differences in trophic enrichment, the interpretation is complicated by $\delta^{15}\text{N}$
343 values also being generally lower in mesic than in xeric habitats (Kelly 2000). Furthermore,
344 presence of grazing livestock may contribute to increased levels of $\delta^{15}\text{N}$ (Kerley and Jarvis,
345 1996). It is possible that there are more grazing animals in the arid areas of Central Asia
346 than in the temperate forest region, contributing to this difference. Consequently, the
347 predictive value of differences in $\delta^{15}\text{N}$ increases in cases when the samples come from the
348 same area, particularly in cases of possible niche overlap. In the case of *blythi* and
349 *halimodendri* there is a possibility that the differences are influenced by other factors than
350 different feeding habits.

351

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

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

368

369 ***Implications of isotopic niche differentiations among other taxa***

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

377

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

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

384

385 $\delta^{15}\text{N}$ values differ significantly between the three southern forms. *halimodendri* on the one
386 hand and *margelanica* and *minula* on the other, where habitat differences are less obvious,
387 and the reason for this is unclear. In contrast, there are no significant differences in $\delta^{15}\text{N}$
388 values between the northern *blythi* and the southern *margelanica* and *minula* in spite of
389 obvious habitat differences (Olsson *et al.*, 2013; Shirihai *et al.*, 2001). Between *blythi* and
390 *althaea* differences in $\delta^{15}\text{N}$ values are nearly statistically significant ($p=0.069$), although the
391 implications of this are unclear.

392

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

396

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

406

407 Loskot (2005) and Williamson (1976) suggested that *minula* and *margelanica* intergrade in
408 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

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

446

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455

456

457

458

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

Allopecies	Site	Country	Status	Year sampled	Year feather grown	Haplotype	Latitude	Longitude	<i>n</i>
<i>althea</i>	Issyk Kol	Kyrgyzstan	On breeding grounds	2003	2002	4	42°25'N	76°70'E	1
<i>althea</i>	Turaigyr	Kazakhstan	On breeding grounds	2003	2002	4	43°29'N	78°38'E	2
<i>althea</i>	Zhetyzhol	Kazakhstan	On breeding grounds	2003	2002	4	43°01'N	76°03'E	1
<i>blythi</i>	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	1	24°16'N	52°52'E	2
<i>blythi</i>	Chokpak	Kazakhstan	Migrating	2002	2001	1	42°31'N	70°38'E	14
<i>blythi</i>	Balkash	Kazakhstan	Migrating	2002	2001	1	46°70'N	74°35'E	1
<i>blythi</i>	Ili River	Kazakhstan	Migrating	2002	2001	1	45°07'N	75°26'E	1
<i>blythi</i>	Sorbulak	Kazakhstan	Migrating	2002	2001	1	43°70'N	76°50'E	3
<i>halimodendri</i>	Al Wathba, Abu	United Arab Emirates	Wintering	2004	2003	2a	24°15'N	54°40'E	3
<i>halimodendri</i>	Al Wathba, Abu	United Arab Emirates	Wintering	2004	2003	2b	24°15'N	54°40'E	2
<i>halimodendri</i>	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	2a	24°16'N	52°52'E	6
<i>halimodendri</i>	Ghantoot, Dubai	United Arab Emirates	Wintering	2004	2003	2b	24°16'N	52°52'E	10
<i>halimodendri</i>	Ili River	Kazakhstan	On breeding grounds or migrating	2003	2003	2a	45°07'N	75°26'E	2
<i>halimodendri</i>	Sorbulak	Kazakhstan	On breeding grounds or migrating	2003	2003	2a	43°70'N	76°50'E	3
<i>halimodendri</i>	Sorbulak	Kazakhstan	On breeding grounds or migrating	2003	2003	2b	43°70'N	76°50'E	1
<i>halimodendri</i>	Hilf	Oman	Wintering	2003	2003	2a	20°66'N	58°90'E	1
<i>halimodendri</i>	Khatmat Milahah	Oman	Wintering	2003	2003	2a	24°95'N	56°35'E	1
<i>halimodendri</i>	Hilf	Oman	Wintering	2003	2003	2b	20°66'N	58°90'E	1
<i>halimodendri</i>	Khatmat Milahah	Oman	Wintering	2003	2003	2b	24°95'N	56°35'E	3
<i>halimodendri</i>	Xinjiang	China	September – Breeding grounds?	2004	2004	2b	42°09'N	89°02'E	1
<i>margelanica</i>	Qinghai	China	On breeding grounds	2003	2002	3	36°30'N	100°60'E	1
<i>margelanica</i>	Xinjiang	China	September – Breeding grounds?	2004	2004	3	42°09'N	89°02'E	4
<i>minula</i>	Xinjiang	China	On breeding grounds	2003	2002	5	40°05'N	81°04'E	9
<i>minula</i>	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) $\delta^2\text{H}$ and (log)
 463 $\delta^{18}\text{O}$, primarily reflecting geographical differences and; (b) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ 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
$\delta^2\text{H}$					
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
$\delta^{18}\text{O}$					
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
$\delta^{13}\text{C}$					
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
$\delta^{15}\text{N}$					
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.

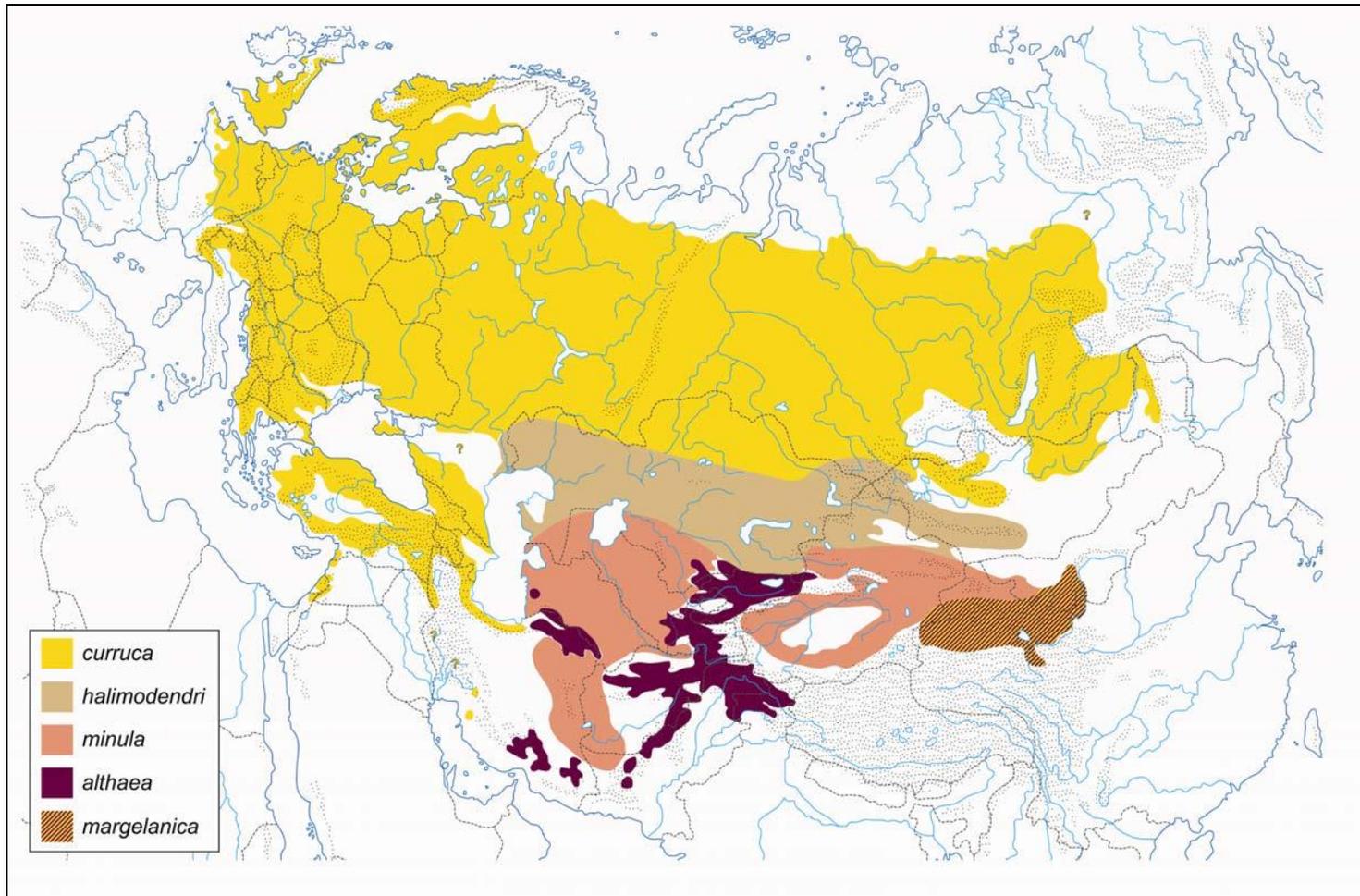


Figure 2. Phylogeny of the Asian Lesser Whitethroat taxa after Olsson *et al.* (2013). The unnamed sister taxon of *halimodendri* is here treated as synonymous with *halimodendri*.

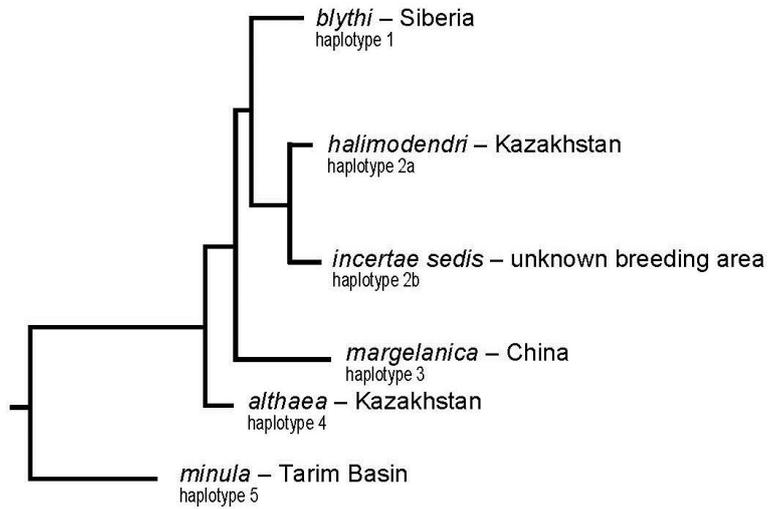
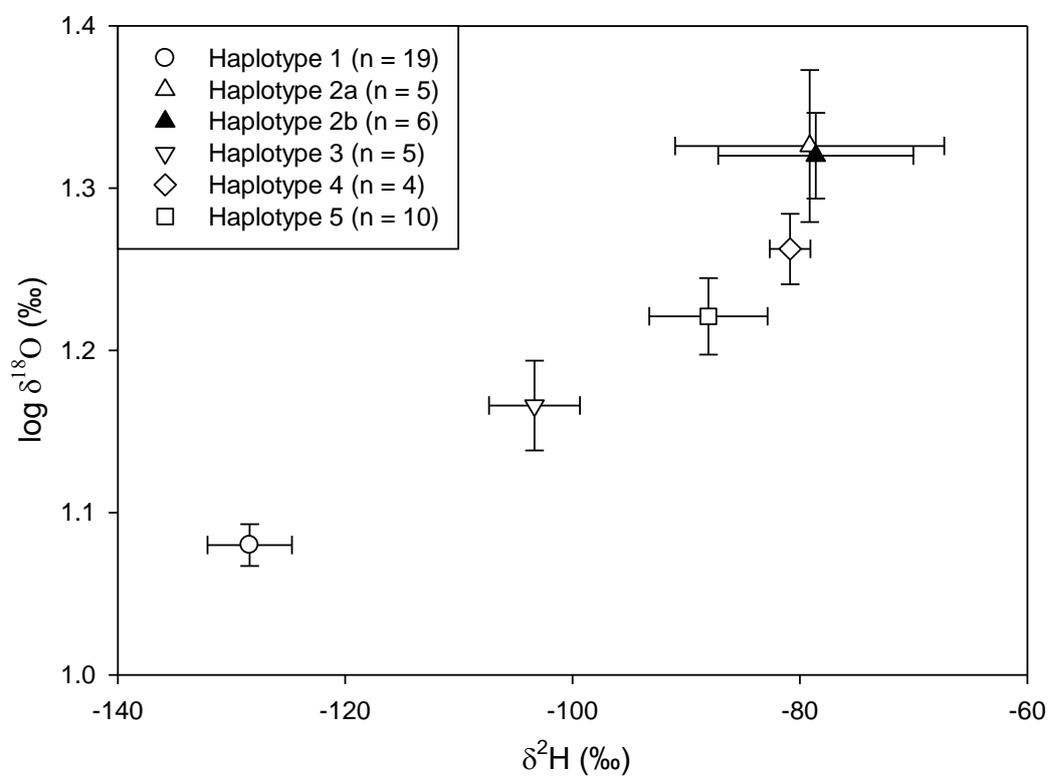
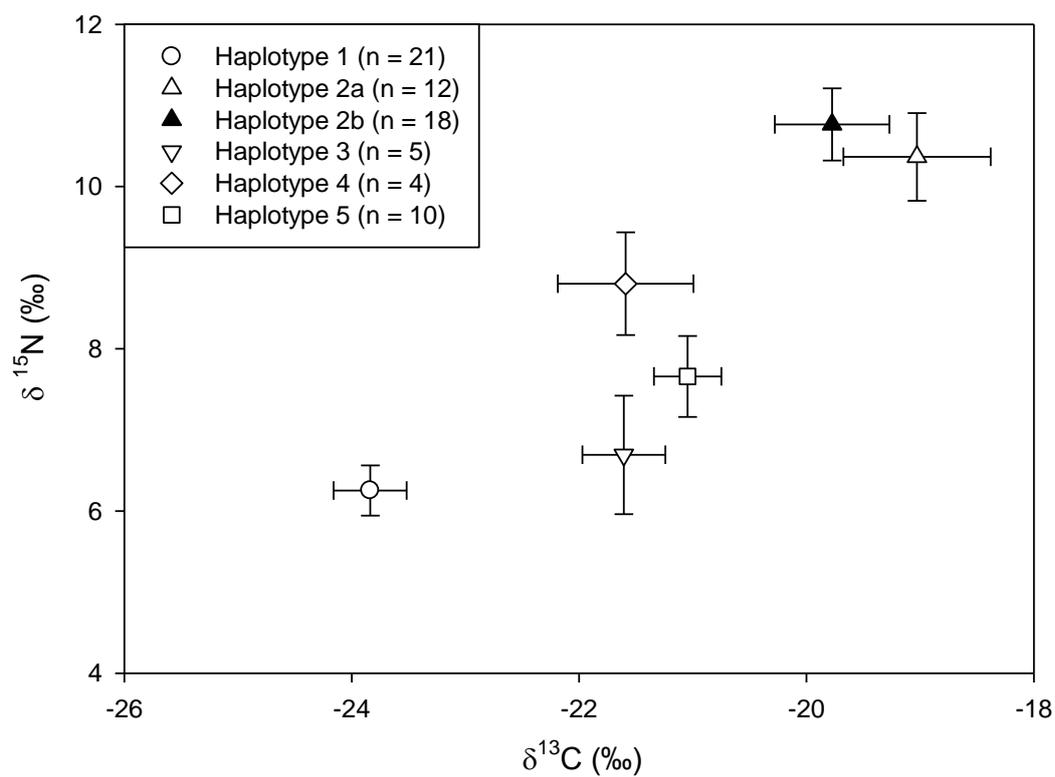


Figure 3 Stable isotopes of hydrogen, carbon, nitrogen and oxygen vary as function of six haplotype groups in the lesser whitethroat complex. Haplotype 1 equals *blythi*, haplotypes 2a and 2b equal *halimodendri*, haplotype 3 equals *margelanica*, haplotype 4 equals *althaea* and haplotype 5 equals *minula*. Values are means ± 1 SE. 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.



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