Title: Divergent mitochondrial and nuclear OXPHOS genes are candidates for genetic incompatibilities in *Ficedula* Flycatchers

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Abstract

Hybrid dysfunction is an important source of reproductive isolation between emerging species. Bateson-Dobzhansky-Muller incompatibilities are theoretically well-recognized as the underlying cause of low hybrid dysfunction. However, especially in wild populations, little empirical evidence exists for which genes are involved in such incompatibilities. The relative role of ecological divergence in causing the build-up of genetic incompatibilities in relation to other processes such as genomic conflict therefore remains largely unknown. Genes involved in energy metabolism are potential candidates for genetic incompatibilities, since energy metabolism depends on co-expression of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) leading to mitonuclear coadaptation. When mitochondrial and nuclear genes lacking a co-evolutionary history appear together in hybrids, incompatibilities could arise. *Ficedula* flycatcher F1 hybrids have a higher resting metabolic rate (RMR) compared to the parental species, which could be a sign of genetic incompatibilities between energy metabolism genes that diverged in response to environmental differences while the species were in allopatry. Based on sequences of 15 mitochondrial genes of 264 individuals, we show that the two species have divergent mtDNA caused by the build-up of mainly synonymous mutations and a few non-synonymous mutations. Pied flycatcher mitogenomes show evidence of non-neutrality, indicating a selective sweep or population expansion. There is little variation in the nuclear OXPHOS-related proteins and no significant deviation from neutrality, however, specific codon identified sites might be under positive selection in both mitochondrial and

31 nuclear genes encoding OXPHOS proteins for complex I and III. Taken together, these diverged
32 mitonuclear genes therefore constitute possible candidates underlying, at least part of the
33 genetic incompatibilities that cause hybrid dysfunction in crosses between collared and pied
34 flycatchers.

Introduction

Sterility or inviability is often observed in the F1 hybrids of genetically diverged populations. Bateson (1909), Dobzhansky (1936) and Muller (1940; 1942) outlined theoretical models explaining the evolution of such genetic incompatibilities in hybrids. They proposed a two-loci system with interacting genes where alternative new mutations can go to fixation in geographically separated populations without causing incompatibilities within any one of the populations (i.e. Bateson-Dobzhansky-Muller incompatibilities; BDMI). However, when the two populations come into secondary contact and interbreed, these alternative alleles can cause hybrid dysfunction as a result of epistasis. Empirical evidence for BDMI has been found in model species such as *Mimulus* (Fishman & Willis 2001) and *Drosophila* (Brideau et al 2006),but general processes that lead to BDMIs are not well described in wild systems. To what extent ecological divergence, rather than other processes such as genomic conflict, is causing the build-up of genetic incompatibilities therefore remains largely unknown.

One set of genes that are potentially interesting candidates for BDMI are those directly involved in energy metabolism(Burton & Barreto 2012, Gershoni et al 2009). The fitness of an organism depends upon efficient energy metabolism under the environmental conditions experienced (e.g. (Bozinovic et al 2011, Mishmar et al 2003). Thus local adaptations to climate can cause rapid divergence between populations (Qvarnström et al 2016). Energy production is regulated by the mitochondria via the oxidative phosphorylation pathway (OXPHOS). Mitochondrial DNA (mtDNA) codes in most organisms for 13 proteins and several non-coding RNAs (transfer RNA, ribosomal RNA). OXPHOS is built up of protein complexes composed of both mitochondrial and nuclear encoded proteins and thus mtDNA and nuclear (nDNA) products strongly interact in this pathway(Smeitink et al 2004). This results in selection for coadaptation between these two genetic systems (Gershoni et al 2009, Willett & Burton 2001). MtDNA mutates quickly (Brown et al 1979) and usually does not recombine, which, in combination with a small effective population size, may lead to fixation of slightly deleterious mutations. Coevolution can then lead to compensatory changes in the interacting nDNA (Rand et al 2004). If energy metabolism in two populations has adapted to different climates (e.g. changes in mtDNA and the interacting nDNA), hybridisation can result in incompatibilities between the OXPHOS proteins in the F1 offspring i.e. BDMIs.

Natural hybrid zones provide good opportunities to study the possible effect of divergent energy metabolism on postzygotic isolation. *Ficedula hypoleuca* and *Ficedula*

67 *albicollis* (pied and collared flycatchers, respectively) are two species of small migratory birds
68 that diverged less than one million years ago (Nadachowska-Brzyska et al 2013) and are
69 frequently used as models for research on ecology and evolution (Qvarnström et al 2010). Pied
70 flycatchers are present mostly in the north of Europe, whereas collared flycatchers have a
71 more southern breeding distribution (Figure 1). In the 1960's, collared flycatchers colonized
72 the Swedish island Öland that was already inhabited by pied flycatchers (Qvarnström et al
73 2010), thus leading to secondary contact between the two species. Divergent climate
74 adaptation seems to play an important role in mitigating the effects of competitive interactions
75 between the two species of flycatchers (Qvarnström et al 2016). Collared flycatchers displace
76 pied flycatchers from the preferred breeding sites (Vallin et al 2012), but appear to be
77 constrained to a narrower niche use, whereas pied flycatchers are less affected by a mismatch
78 between food abundance and nestling growth (Qvarnström et al 2009, Sirkiä et al 2018). This
79 robustness might be explained by higher plasticity in metabolic rate of pied flycatchers, as pied
80 flycatcher nestlings have a lower resting metabolic rate (RMR) at higher temperatures
81 (associated with low food availability) and increased RMR in response to relaxed sibling
82 competition when compared to collared flycatchers (McFarlane et al 2018). The flexible RMR
83 response to environmental conditions observed in pied flycatchers may allow pied flycatchers
84 to breed in low quality habitat and late in the season when food availability is lower
85 (McFarlane et al 2018).

86 Despite competition between the two species over similar nesting sites, they also
87 interbreed at a low frequency, resulting in hybrids with an intermediate plumage phenotype
88 (Figure 1). Hybrids display extremely low fertility as female hybrids lay empty eggs (Svedin et
89 al 2008) and male hybrids have malformed sperm (Ålund et al 2013). Additionally, flycatcher
90 hybrids have a higher RMR compared to both parental species (McFarlane et al 2016),
91 suggesting increased energy requirements for maintenance and thus likely impacting fitness
92 (i.e. less available energy for other traits). This might indicate genetic incompatibilities in the
93 mitonuclear pathway, however, it remains unknown to what extent the two species have
94 diverged in their mtDNA and corresponding nuclear OXPHOS genes.

95 In this study we aimed to investigate if genetic divergence of the mtDNA and associated
96 nuclear OXPHOS genes between collared and pied flycatchers could play a role in hybrid
97 dysfunction. We examined patterns of neutrality and selection on both types of DNA, to narrow

98 in on regions that may be associated with differences in plasticity of RMR and/or be causing
99 BDMIs between the two species.

100

101 **Materials and methods**

102 *Population monitoring*

103 The flycatcher population on Öland has been continuously studied since 2002 (see
104 (Qvarnström et al 2009) for a thorough description of our field methods). Briefly, more than
105 2000 nestboxes are monitored for breeding activity in May and June. All birds (male and
106 female adults, and nestlings) are individually ring marked and blood samples are taken for
107 genetic analyses. mtDNA was sequenced from 227 individuals. Additionally, whole genome
108 DNA data has previously been generated for 38 individuals (Burri et al 2015). All research on
109 flycatchers was approved by the Linköping Animal Ethics Committee.

110

111 *Bioinformatics*

112 We sequenced the 12S, 16S, ATP, CO1, CO3, CytB, ND1, ND2, ND3, ND4L, ND4 and ND6 regions
113 of the mtDNA of 227 flycatchers (165 collared flycatchers, 45 pied flycatchers, 17 hybrids) with
114 paired Sanger sequencing using the primers from (Amer et al 2013).

115 All sequences were trimmed with DNA Baser v4.36.0 (BioSoft 2013), on default settings
116 for samples with 'normal' quality (trim until >60% good bases (QV>20) in 16 base window).
117 Paired reads were assembled into contigs using the DNA Baser default settings and visually
118 inspected for ambiguities in the forward versus the reverse strand. We obtained good quality
119 sequences for different individuals per gene, resulting in different numbers of sequences per
120 gene alignment.

121 For the nuclear analysis we used genomic sequences of 19 pied and 19 collared
122 flycatchers from Öland (available on EMBL-EBI European Nucleotide Archive (accession
123 PRJEB7359;Burri et al 2015). Exons of the OXPHOS-related genes were extracted from the
124 genomic data aligned to the collared flycatcher reference (FicAlb1.5) using the annotated
125 genome (we extracted both nuclear and mitochondrial genes and merged the data for the
126 mitochondrial genes with the new sequence data). Pseudo-haploid consensus sequences of the

genes were made by calling all positions covered by at least two reads and randomly choosing an allele for heterozygous sites using ANGSD –dofasta (Korneliussen et al 2014). An overview of all the included genes (nuclear and mitochondrial) can be found in Supplementary Table S1. We included all mitochondrial genes and most nuclear OXPHOS-related genes that are a part of OXPHOS complexes I, III, IV and V (complex II is not included, since this complex only contains nuclear-encoded genes and thus has no direct interactions with mitochondrial genes). One pied individual was excluded from our analysis as it clustered with collared flycatchers based on the mtDNA, suggesting potential contamination or mislabelling.

All individual contigs were aligned with AliView using MUSCLE (default settings; Larsson 2014). The collared flycatcher mitochondrial genome (Ekblom et al 2014;NCBI) and nuclear gene sequences from NCBI were used as reference sequences. For mtDNA, all insertions, deletions and polymorphisms in the sequences were visually inspected with DNA Baser to ensure no base calling mistakes.

Haplotype Networks

To visualise inter- and intraspecific genetic variation we made two haplotype networks, one based on mtDNA and one based on nDNA. SequenceMatrix 1.8 (Vaidya et al 2011) was used to concatenate the genes per individual and PopART (Bandelt et al 1999, Leigh & Bryant 2015) was used to make a median-joining haplotype network of the concatenated sequences. PopART only includes sites called in >95% of individuals, so we based the mtDNA network on a concatenation of the regions for which sequences were available for most individuals (12S, ATP, ND1, ND2, ND3 and ND4; Bandelt et al 1999, Leigh & Bryant 2015). In addition, we removed individuals that had few called bases from the analysis; the resulting haplotype network consists of 147 collared, 12 hybrid and 29 pied flycatchers. For nDNA enough sequence data was available to include all genes and individuals.

Estimates of diversity and divergence

We calculated haplotype diversity (H_d) and nucleotide diversity (π) as measures of genetic diversity within each species, separately for mtDNA and nuclear OXPHOS-related genes. In addition, divergence between the two species was analysed by calculating the

157 average number of nucleotide substitutions per site between populations with a Jukes-Cantor
158 adjustment (D_{XY}) and the average number of nucleotide differences between the populations
159 (k). All analyses were done with DnaSP (version 6.12; Rozas et al 2017), on concatenated
160 alignments of the genes.

161 mtDNA summary statistics were calculated for a concatenated alignment of the regions
162 for which we obtained the most sequence data (12S, 16S, ATP, CO1, CO3, CytB, ND1, ND2, ND3,
163 ND4). In order to avoid biased results due to different sample sizes we used the 23 pied and 23
164 collared individuals for which the most complete sequence dataset was available.

165 We identified and classified polymorphic sites in our alignments. A site was considered
166 as a polymorphic site when the polymorphism was shared by either at least five individuals
167 (when the alignment contained both newly sequenced mtDNA data and data extracted from
168 (Burri et al 2015; ±264 individuals), or shared by at least three individuals (when an alignment
169 only contained data extracted from (Burri et al 2015; ±37 individuals). Per site, we classified
170 the polymorphism as a fixed difference, as variable in either species or as variable in both
171 species, and we determined whether the site is non-coding, non-synonymous or synonymous.

172 Since variation in nDNA consisted for a large part of shared polymorphisms (see
173 Results), we tested whether there was a significant difference in allele frequency for shared
174 SNPs between the two species. Shared mitochondrial SNPs were compared between
175 haplogroups, with mtDNA sequences for the hybrids added to the data of the corresponding
176 maternal species (haplogroups are based on haplotype network and known species
177 information; see Results). We applied a chi-squared test (Ryman et al 2006) and Fisher's exact
178 test, which is intended for small sample sizes (Cammen et al 2015, Ryman & Jorde 2001). We
179 used a Bonferroni correction to adjust for multiple testing. We report Fisher's exact test below,
180 and the chi-squared test in Supplementary Table S2. This analysis was done in R (R Core Team
181 2017; scripts available in supplementary material).

182

183 *Analyses of non-neutrality and selection*

184 Fu and Li's F and D test statistic (FLF*, FLD*; Fu & Li 1993) and Tajima's D (Tajima
185 1989) were calculated with DnaSP to test departures from neutrality in all genes of interest. A
186 negative neutrality test can be a sign of purifying selection, selective sweep or population

187 expansion, whereas a positive neutrality test can be a sign of balancing selection or a
188 bottleneck.

189 We used a variety of methods to identify positive selection on our genes of interest. To
190 identify codon specific positive selection, based on the ratio of non-synonymous versus
191 synonymous substitutions per site (ω; dN/dS), we used CodeML (from the package PAML;
192 Yang 2007) which uses a Bayesian Empirical Bayes-method (BEB) to identify positively
193 selected sites. Next, we employed a likelihood ratio test to compare a model with a single ω
194 (model 0) to models with variable ω (either nearly neutral or discrete). Subsequently,
195 likelihood-values obtained for models including positive selection were compared to the values
196 for models without positive selection (positive selection vs nearly neutral and beta and omega
197 vs beta). Additionally, we used the 'mixed effects model of evolution' (MEME; Murrell et al
198 2012) and 'fast unconstrained Bayesian approximation' (FUBAR; Murrell et al 2013) from
199 HYPHY (datamonkey.org; Kosakovsky Pond et al 2005, Weaver et al 2018). MEME uses
200 maximum likelihood estimation to identify episodic selection on individual sites (Murrell et al
201 2012). FUBAR uses a Bayesian approach to infer non-synonymous and synonymous
202 substitution rates per site (Murrell et al 2013).

203

204 **Results**

205 *Haplotype network*

206 We confirmed that the two species have diverged on both the mt- and nDNA as they
207 cluster separately in the haplotype-networks (Figures 2, 3). Based on the mtDNA network, we
208 separated the two species into haplogroups using the pied and collared clusters, and assigned a
209 maternal species to all hybrids. Six hybrids clustered with pied flycatchers, suggesting pied
210 flycatcher maternity, and six hybrids clustered with collared flycatchers, suggesting collared
211 flycatcher maternity (Figure 2). Additionally, three 'collared hybrids' and two 'pied hybrids' did
212 not have enough sequences available for the haplotype network analysis, so are not included.
213 Within both clusters, most haplotypes differed from each other by a few mutations, as
214 indicated by the perpendicular bars on the edges. The collared flycatcher cluster had 17
215 haplotypes that were shared between several individuals, whereas in the pied flycatcher
216 cluster most individuals had unique haplotypes (only 3 haplotypes are shared). The collared

217 NCBI reference sequence was a part of the circle CF-10 which is the most common haplotype
218 among collared flycatchers.

219 Similarly, pied and collared flycatchers cluster separately based on the nuclear
220 haplotypes of OXPHOS-related genes (figure 3). Unsurprisingly, each individual had a unique
221 haplotype, separated by several mutations from all other individuals.

222

223 *Genetic diversity and divergence*

224 Mt- and nDNA showed slightly different patterns of genetic diversity and divergence.
225 We found higher genetic and haplotype diversity in pied flycatcher mtDNA (Hd = 1, π_{pied} =
226 0.00162) versus collared flycatcher mtDNA (Hd = 0.976, $\pi_{collared}$ = 0.00148), whereas nDNA
227 was more diverse in collared flycatchers (π_{pied} = 0.00269, SD = 0.00008; $\pi_{collared}$ = 0.00280, SD
228 = 0.00008 (NS); Table 1). As expected nDNA is generally less diverged compared to mtDNA.
229 Mitochondrial divergence between the two species measured as the absolute divergence (D_{XY})
230 and average number of nucleotide differences between the two populations (k; Table 1) was
231 0.03302 (SD = 0.00244) and 175.745 respectively. Divergence between the nDNA of the two
232 species was 0.00422 (D_{XY} , SD = 0.00031; Table 1).

233

234 *Polymorphic sites*

235 We find clear differences between the polymorphic sites in the nDNA and the mtDNA.
236 There are fewer polymorphic sites in the nuclear OXPHOS-related genes (N = 118) compared
237 to the mtDNA (N = 454) and more fixed differences between the species in the mtDNA (N =
238 188) compared to the nDNA (N = 2).

239 In the mtDNA, we identified 454 variable sites (out of 11,413 sites). 188 of those were
240 fixed differences, as well as 28 shared polymorphisms and 238 polymorphisms that were
241 monomorphic in one species but polymorphic in the other (Table S1). The majority of variable
242 sites were in the third codon position of the nucleotide sequence and were synonymous
243 (392/454), but we identified 62 non-synonymous polymorphisms. 13 of the non-synonymous
244 SNPs were fixed between the two species (in ND1, ND2, ND4L, ND5, CO2 and ATP6), and a
245 large proportion of non-synonymous polymorphisms were shared between the two species
246 (12/28). 17 polymorphic non-synonymous mutations were polymorphic in the pied but

247 monomorphic in the collared flycatchers, and 20 non-synonymous mutations were
248 polymorphic in the collared but monomorphic in the pied flycatchers (Table S1).

249 In the coding regions of the nDNA we identified 118 variable sites (out of 20,868 sites).
250 Many of the analysed genes did not have any variation in the coding region (Table S1). There
251 were only two fixed differences between the two species in the coding regions of the analysed
252 genes (both in COX7C) and five in the non-coding parts surrounding genes that were included
253 in the alignments (ATP5F1B, ATP5MG; Table S1). Generally, polymorphisms were shared
254 between species (151, 46 in coding regions) although 147 (47 in coding regions) were
255 polymorphic only in collared flycatchers and 109 (22 in coding regions) were polymorphic
256 only in pied flycatchers. When the OXPHOS complexes were compared, eight non-synonymous
257 mutations were present in complex I, five in complex III, no non-synonymous mutations in
258 complex IV and one in complex V. Most non-synonymous mutations were found in collared
259 flycatchers, with the exception of the mutation in complex V and one mutation in complex III
260 that were only polymorphic in pied flycatchers, while five non-synonymous polymorphisms
261 were shared between the two species.

262 Since many SNPs in the nDNA were shared between the two haplogroups, we calculated
263 allele frequency differences to test for species divergence. All 179 shared SNPs (151 nDNA, 28
264 mtDNA) were analysed with a Fisher's exact test and a chi-squared test (Figure 4 and Table
265 S2). 25 SNPs were significantly different between the species, of which 15 were mtDNA and 10
266 nDNA. Most significant SNPs were a part of complex I (14), followed by complex IV (6), III (3)
267 and V (2). Chi-squared tests gave very similar results, although one site in nDNA complex I was
268 no longer significantly different (Table S2). This result indicates that even though there are
269 fewer shared polymorphic sites in mtDNA, those sites are more often significantly different
270 between the species.

271

272 *Neutrality and positive selection*

273 We found deviations from neutrality in the OXPHOS genes in the mtDNA of pied and
274 collared flycatchers, but not in the nDNA of either species, possibly due to low power because
275 there was little to no variation in the coding regions of the nDNA (Li's D and F Statistics, Table
276 2). Specifically, in the mtDNA, we found evidence of selection on 12S, 16S, CytB, ATP6 (both
277 species) and ND2, ND4, ND6, CO1, CO2 and CO3 (only pieds), possibly indicating a selective

278 sweep. Alternatively, a recent population expansion might be the cause of the observed
279 deviations from neutrality (Table 2). There was no significant pattern of selection on the other
280 genes and the pattern was consistent between Fu and Li´s D and Tajima´s D for almost all genes
281 (Table 2).

282 When we analysed the mtDNA genes as a concatenated alignment, FLD*, FLF* and
283 Tajima's D were significantly negative for the pied population, but not different from zero in
284 the collared population (Table 2). For the concatenated nDNA OXPHOS regions, the neutrality
285 tests for the collared flycatchers were more negative compared to the pied flycatchers
286 (collared: FLF* = -0.3591, FLD* = -0.4120, Tajima's D = -0.3404; pied: FLF* = -0.2638, FLD* = -
287 0.2290, Tajima's D = -0.0391), although none of the tests is significantly negative.

288 In addition to examining neutrality in each gene, we also identified positive selection on
289 specific codons in both mtDNA and nDNA for complexes I and III. In the mitochondrial genes,
290 we found signals of positive selection on 14 sites in ND1, ND4, CytB, CO1 and CO3 (Table 3).
291 These genes are part of complexes I, III and IV. Further, we found positive selection between
292 the species on 12 sites in the nuclear genes NDUFA5, NDUFB3, NDUFS1, NDUFS7, NDUFV1,
293 UQCRC2 and ATP5PF. These genes are a part of the complexes I, III and V.

294

295 **Discussion**

296 Collared and pied flycatchers diverged less than 1 million years ago (Nadachowska-
297 Brzyska et al 2013) with strong postzygotic reproductive isolation in the form of hybrid
298 dysfunction and infertility (Ålund et al 2013, McFarlane et al 2016, Svedin et al 2008). Here, we
299 specifically focus on divergence in mtDNA and the interacting nuclear OXPHOS genes, as we
300 have recently demonstrated that phenotypic differences in metabolic rate play important roles
301 related to both differences in niche breath of the two species (McFarlane et al 2018) and
302 hybrid dysfunction (McFarlane et al 2016). Using data from free-living collared, pied and
303 hybrid flycatchers, we demonstrate distinct divergence in both the mtDNA (Figure 2) and in
304 OXPHOS-related nDNA (Figure 3) of these species. Both mtDNA and nDNA divergence are
305 significantly higher than the genome wide median divergence previously estimated for this
306 species pair (mtDNA D_{XY} = 0.033±0.002, nDNA D_{XY} =0.0042±0.0003 compared to 0.00013 from
307 (Ellegren et al 2012). This suggests that both the mitochondrial and nuclear OXPHOS genes
308 have higher levels of divergence between collared and pied flycatchers than the average gene

309 in the genome. This divergence could provide at least some of the fuel for BDMIs between the
310 species affecting hybrid performance.

311 Polymorphisms with different frequencies can also contribute to incompatibilities
312 between species (Cutter 2012). For example, a polymorphic haplotype in *Capsella sp.,* which is
313 maintained by balancing selection, results in both compatible and incompatible hybrid crosses
314 (Sicard et al 2015). Recent work on *Drosophila* suggests that within species incompatibilities
315 are common and are maintained at low frequencies through mutation-selection balance (Pool
316 2015). Incompatibilities will be kept at low frequencies within species, but could result in
317 hybrid sterility or inviability between species if different alleles are preferred in each
318 population. We have demonstrated here polymorphisms (both shared and not shared) in
319 OXPHOS genes (Table S2), which could contribute to incompatibilities between collared and
320 pied flycatchers.

321 While mitochondrial variation was previously thought to be rapidly purged and unlikely
322 to influence phenotypic differences in contemporary populations, recent evidence has
323 suggested that this variation may in fact reflect adaptive processes (Bazin et al 2006).
324 Mitochondria are gene-dense and there is growing evidence for their non-neutral evolution
325 (e.g. (Galtier et al 2009, Lamb et al 2018, Morales et al 2015, Ruiz-Pesini et al 2004). It is
326 difficult to disentangle the effects of selection from the effects of demographic processes using
327 genomic data alone without using phenotypic data to support the role of selection (Hill et al
328 2018, Walsh & Lynch 2018). Our study population of collared flycatchers went through a
329 recent bottleneck as they colonized the island of Öland about fifty years ago followed by a
330 drastic population expansion (Kardos et al 2017). In contrast, pied flycatchers were already
331 present on the island and breed further north as compared to collared flycatchers (Figure 1).
332 We found both higher diversity and negative values of neutrality tests in mtDNA in pied
333 flycatchers (Table 1,2). This is not surprising given the larger starting population as well as the
334 fact that the pied flycatcher population on Öland is not isolated to the same extent as the
335 collared flycatcher population and hints at selection rather than demography leading to these
336 patterns. This relatively higher diversity is consistent with the observed adaptive plasticity in
337 pied flycatcher RMR and could be the result of a better adaption to the variable northern
338 climate (McFarlane et al 2018). We interpret the marked divergence in OXPHOS-related genes
339 (including fixation of different non-synonymous mutations in mtDNA) together with signs of
340 positive selection in both mitochondrial and nuclear genes encoding OXPHOS proteins for

341 complex I and III as a legacy of different climate adaptation from the recent time in allopatry
342 (Qvarnström et al 2016). We found signals of selection associated with the OXPHOS complex I
343 in both the mitochondrial and nuclear genomes (Table 2), including evidence for positive
344 selection in ND4 and ND5 (Table 3). A similar pattern was recently found in Australian Eastern
345 Yellow Robins (*Eopsaltria australis*), where there is a cline of mitonuclear lineages that quickly
346 diverges (relative to dispersal distance) in a region of climatic transition, suggesting a link
347 between mtDNA haplotype and environment in this species (Morales et al 2018). Moreover, a
348 comprehensive meta-analysis identified ND5 as a common site of positive selection across
349 metazoan lineages (Garvin et al 2015). Taken together, this suggests that the pattern that we
350 have documented, especially in ND5, may indicate adaptive changes related to environmental
351 variation.

352 Since the process of mitonuclear co-adaptation likely depends on climate (Dowling et al
353 2008), a logical prediction is that interbreeding between populations that are adapted to
354 different climates may result in hybrid individuals with mismatched mitonuclear genes (Chou
355 & Leu 2010). Mitonuclear mismatches have recently been hypothesized to drive reproductive
356 isolation in the context of avian speciation processes (Hill 2017), but empirical evidence is rare.
357 Some studies on other organisms show that poorly functioning mitonuclear interactions may
358 lead to various forms of dysfunction ranging from increased context-dependent metabolic
359 costs (Arnqvist et al 2010, Hoekstra et al 2013) to reduced fertility and viability of hybrids
360 (Breeuwer & Werren 1995, Ellison & Burton 2010). These studies were all based on
361 experimental crosses where effects of maternal mtDNA were tested against fully co-evolved
362 versus non-coevolved nuclear background. Since introgression against a new nuclear
363 background occurs stepwise in nature, a more likely scenario is that F1 hybrids with only
364 partially mismatched mitonuclear genomes are already exposed to selection. This is the case
365 with the sampled F1 *Ficedula* hybrids where mtDNA occurs together with parts of the co-
366 evolved nuclear genome. If there could be future generations of backcrossing in flycatchers,
367 then patterns of non-random mitonuclear co-introgression would be expected to be seen as
368 found in Australian Eastern Yellow Robins (*Eopsaltria australis*; Morales et al 2018). These
369 robins range across coastal and inland habitats on the southwest coast of Australia, including
370 across variable temperature and precipitation gradients (Morales et al 2018). The underlying
371 expectation is that backcrossed individuals with mitonuclear mismatches are continuously
372 removed by selection. A large region of Chromosome 1A, associated with OXPHOS complex I,

373 has co-introgressed with the associated mitolineage into the contact zone between coastal and
374 inland populations (Morales et al 2018). This suggests that mitolineage is associated with
375 climate adaptation in robins, a hypothesis that would be strengthened by the examination of
376 the association between phenotypic data such as metabolic rate and these co-introgressing
377 lineages (Hill et al 2018).

378 There are reported cases of metabolic dysfunction in hybrid birds, in addition to what
379 has been reported in flycatchers. Specifically, captive stone chat hybrids (*Saxicola torquata*
380 *ssp.)* with mismatched mtDNA and nuclear DNA had different RMR from the parental types
381 (Tieleman et al 2009), and wild caught black-capped x Carolina chickadees (*Poecile atricapillus*
382 and *P. carolinensis)* had higher mass-corrected RMR than the parental species (Olson et al
383 2010). When taken together, the patterns of disrupted metabolic rate in hybrid flycatchers
384 (McFarlane et al 2016), the divergence that we have documented in the current study and
385 hybrid sterility in *Ficedula* F1 individuals (Ålund et al 2013, Svedin et al 2008) suggest that
386 mismatched OXPHOS interactions may have a functional effect on hybrid fitness.

387 Mitonuclear interactions are a likely candidate for BDMI incompatibilities across a
388 variety of taxa (Burton and Barreto 2012), and our results are in line with this suggestion.
389 OXPHOS genes comprise a well characterized, highly conserved gene network (Rolfe & Brown
390 1997, van den Heuvel & Smeitink 2001), that nonetheless has higher than expected levels of
391 divergence between collared and pied flycatchers. This is consistent with the high level of
392 reproductive isolation between the two flycatcher species, which have near complete
393 postzygotic isolation after a short period of divergence. In contrast, the average hybridizing
394 avian species pair still produces fertile hybrids until approximately 7 million years of
395 divergence (Price & Bouvier 2002). While we have zoomed in on possible candidates for
396 incompatibilities in this study, we have not yet found specific mitonuclear BDMIs between the
397 two *Ficedula* species. The polymorphisms that we found, including those shared between the
398 species, are candidates for inter-specific incompatibilities

399 If polymorphic incompatibilities resulting in BDMIs were driving hybrid infertility in *Ficedula*
400 hybrids, then variation in fertility and/or RMR in the F1 generation could be associated with
401 variation among mismatched mitonuclear genotypes to pinpoint specific BDMIs. Additionally,
402 we might expect to find some fertile hybrids, as has been documented both in the above
403 *Capsella* hybrids as well as in *Mus musculus musculus* x *M. m. domesticus* where polymorphic

404 incompatibilities have also been documented (Larson et al 2018). However, all sampled
405 flycatcher hybrids appear to be infertile (Ålund et al 2013, Svedin et al 2008) suggesting that if
406 there are compatible haplotypes at these loci, they are rare. In order to specify potential
407 specific mitonuclear BDMIs caused by fixed differences between the two *Ficedula* species, we
408 would need to use genomic methods that could restore hybrid fitness such as laboratory
409 methods that would allow us to have two copies of candidate alleles against an otherwise F1
410 background. This is, however, beyond the scope of the current study.

411 At late stages of speciation (i.e. when postzygotic isolation is complete or nearly complete), it is
412 difficult to tell whether specific incompatibilities have evolved before or after reproductive
413 isolation (Seehausen et al 2014), as BDMIs tend to 'snowball'. Theoretically, it may only take
414 one epistatic interaction stemming from divergence while in allopatry to lead to complete
415 reproductive isolation (Bateson 1909, Dobzhansky 1936, Muller 1940, 1942). After isolation is
416 complete, other incompatibilities can accumulate, but these are a consequence rather than a
417 cause of reproductive isolation, leading to the build-up of tens or hundreds of genes involved in
418 incompatibilities (Presgraves 2003). For example, hundreds of genes affect hybrid
419 incompatibilities between recently diverged swordtail species (*Xiphophorus birchmanni* and *X.*
420 *malinche*; Schumer et al 2014). While it seems possible that the divergence in OXPHOS genes is
421 due to differences in climate adaptation between the flycatcher species (Qvarnström et al
422 2016), it is premature to conclude whether mitonuclear incompatibilities are causing
423 postzygotic isolation between the two species.

424 Here, we demonstrate higher than expected divergence between collared and pied flycatchers
425 in the OXPHOS genes, hinting that these genes may be contributing to the hybrid metabolic
426 dysfunction previously documented, possibly via BDMIs. We also found evidence of recent
427 selection, particularly in the mtDNA gene ND5 which is part of the OXPHOS complex I and
428 appears to be diverging between collared and pied flycatchers, consistent with selection found
429 in other avian studies. Taken together, this suggests that metabolic dysfunction resulting from
430 mt-nDNA incompatibilities may be a factor contributing to hybrid dysfunction in this system.

431

432 **Author Contributions**:

SEM and AQ conceived and designed the study and collected the data. Evdh and TvdV performed analyses. Evdh, SEM and AQ wrote the manuscript and all authors contributed to meaningful discussions and revisions of the manuscript.

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443 **References:**

444 Ålund M, Immler S, Rice AM, Qvarnström A. 2013. Low fertility of wild hybrid male flycatchers
445 despite recent divergence. *Biology letters* 9: 20130169
446 Amer SAM, Ahmed MM, Shobrak M. 2013. Efficient newly designed primers for the
447 amplification and sequencing of bird mitochondrial genomes. *Bioscience, Biotechnology,*
448 *and Biochemistry* 77: 577-81
449 Arnqvist G, Dowling DK, Eady P, Gay L, Tregenza T, et al. 2010. Genetic architecture of
450 metabolic rate: environment specific epistasis between mitochondrial and nuclear
451 genes in an insect. *Evolution* 64: 3354-63
452 Bandelt H-J, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific
453 phylogenies. *Molecular Biology and Evolution* 16: 37-48
454 Bateson W. 1909. Heredity and variation in modern lights. *Darwin and modern science*
455 Bazin E, Glémin S, Galtier N. 2006. Population size does not influence mitochondrial genetic
456 diversity in animals. *Science* 312: 570-72
457 BioSoft H. 2013. DNA Sequence Assembler v4.
458 Bozinovic F, Calosi P, Spicer JI, Bozinovic F, Calosi P, Spicer JI. 2011. Physiological correlates of
459 geographic range in animals. *Annual review of ecology, evolution and systematics* 42:
460 155-79
461 Breeuwer JA, Werren JH. 1995. Hybrid breakdown between two haplodiploid species: the role
462 of nuclear and cytoplasmic genes. *Evolution* 49: 705-17
463 Brideau NJ, Flores HA, Wang J, Maheshwari S, Wang X, Barbash DA. 2006. Two Dobzhansky-
464 Muller genes interact to cause hybrid lethality in Drosophila. *Science* 314: 1292-95
465 Brown WM, George M, Jr., Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA.
466 *Proceedings of the National Academy of Sciences* 76: 1967-71
467 Burri R, Nater A, Kawakami T, Mugal CF, Olason PI, et al. 2015. Linked selection and
468 recombination rate variation drive the evolution of the genomic landscape of
469 differentiation across the speciation continuum of Ficedula flycatchers. *Genome research*
470 25: 1656-65
471 Burton RS, Barreto FS. 2012. A disproportionate role for mtDNA in Dobzhansky-Muller
472 incompatibilities? *Molecular ecology* 21: 4942-57

473 Cammen KM, Schultz TF, Rosel PE, Wells RS, Read AJ. 2015. Genomewide investigation of
474 adaptation to harmful algal blooms in common bottlenose dolphins (Tursiops
475 truncatus). *Molecular Ecology* 24: 4697-710
476 Chou JY, Leu JY. 2010. Speciation through cytonuclear incompatibility: insights from yeast and
477 implications for higher eukaryotes. *Bioessays* 32: 401-11
478 Cutter AD. 2012. The polymorphic prelude to Bateson–Dobzhansky–Muller incompatibilities.
479 *Trends in ecology & evolution* 27: 209-18
480 Dobzhansky T. 1936. Studies of hybrid sterility. II. Localization of sterility factors in Drosophila
481 pseudoobscura hybrids. *Genetics* 21: 113-35
482 Dowling DK, Friberg U, Lindell J. 2008. Evolutionary implications of non-neutral mitochondrial
483 genetic variation. *Trends in ecology & evolution* 23: 546-54
484 Ekblom R, Smeds L, Ellegren H. 2014. Patterns of sequencing coverage bias revealed by ultra-
485 deep sequencing of vertebrate mitochondria. *BMC Genomics* 15: 1-9
486 Ellegren H, Smeds L, Burri R, Olason PI, Backström N, et al. 2012. The genomic landscape of
487 species divergence in Ficedula flycatchers. *Nature* 491: 756-60
488 Ellison C, Burton R. 2010. Cytonuclear conflict in interpopulation hybrids: the role of RNA
489 polymerase in mtDNA transcription and replication. *Journal of evolutionary biology* 23:
490 528-38
491 Fishman L, Willis JH. 2001. Evidence for Dobzhansky-Muller incompatibilities contributing to
492 the sterility of hybrids between *Mimulus gattatus* and *M. nastus Evolution* 55: 1932-42
493 Fu Y-X, Li W-H. 1993. Statistical tests of neutrality of mutations. *Genetics* 133: 693-709
494 Galtier N, Nabholz B, Glémin S, Hurst GDD. 2009. Mitochondrial DNA as a marker of molecular
495 diversity: a reappraisal. *Molecular Ecology* 18: 4541-50
496 Garvin MR, Bielawski JP, Sazanov LA, Gharrett AJ. 2015. Review and meta-analysis of natural
497 selection in mitochondrial complex I in metazoans. *Journal of Zoological Systematics and*
498 *Evolutionary Research* 53: 1-17
499 Gershoni M, Templeton AR, Mishmar D. 2009. Mitochondrial bioenergetics as a major motive
500 force of speciation. *Bioessays* 31: 642-50
501 Hill GE. 2017. The mitonuclear compatibility species concept. *The Auk* 134: 393-409
502 Hill GE, Havird JC, Sloan DB, Burton RS, Greening C, Dowling DK. 2018. Assessing the fitness
503 consequences of mitonuclear interactions in natural populations. *Biological Reviews*

504 Hoekstra LA, Siddiq MA, Montooth KL. 2013. Pleiotropic Effects of a Mitochondrial–Nuclear
505 Incompatibility Depend upon the Accelerating Effect of Temperature in Drosophila.
506 *Genetics* 195: 1129-39
507 Kardos M, Qvarnström A, Ellegren H. 2017. Inferring Individual Inbreeding and Demographic
508 History from Segments of Identity by Descent in Ficedula Flycatcher Genome
509 Sequences. *Genetics* 205: 1319-34
510 Korneliussen TS, Albrechtsen A, Nielsen R. 2014. ANGSD: analysis of next generation
511 sequencing data. *BMC bioinformatics* 15: 356
512 Kosakovsky Pond SL, Frost SDW, Muse SV. 2005. HyPhy: Hypothesis testing using phylogenies.
513 *Bioinformatics* 21: 676-79
514 Lamb AM, Gan HM, Greening C, Joseph L, Lee Yin P, et al. 2018. Climate-driven mitochondrial
515 selection: A test in Australian songbirds. *Molecular Ecology* 27: 898-918
516 Larson EL, Vanderpool D, Sarver BA, Callahan C, Keeble S, et al. 2018. The evolution of
517 polymorphic hybrid incompatibilities in house mice. *Genetics* 209: 845-59
518 Larsson A. 2014. AliView: A fast and lightweight alignment viewer and editor for large datasets.
519 *Bioinformatics* 30: 3276-78
520 Leigh JW, Bryant D. 2015. POPART: Full-feature software for haplotype network construction.
521 *Methods in Ecology and Evolution* 6: 1110-16
522 McFarlane SE, Ålund M, Sirkiä PM, Qvarnström A. 2018. Difference in plasticity of resting
523 metabolic rate–the proximate explanation to different niche breadth in sympatric
524 Ficedula flycatchers. *Ecology and Evolution* 8: 4575-86
525 McFarlane SE, Sirkiä PM, Ålund M, Qvarnström A. 2016. Hybrid Dysfunction Expressed as
526 Elevated Metabolic Rate in Male Ficedula Flycatchers. *PLOS ONE* 11: e0161547
527 Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, et al. 2003. Natural selection shaped
528 regional mtDNA variation in humans. *Proceedings of the National Academy of Sciences*
529 100: 171-76
530 Morales HE, Pavlova A, Amos N, Major R, Kilian A, et al. 2018. Concordant divergence of
531 mitogenomes and a mitonuclear gene cluster in bird lineages inhabiting different
532 climates. *Nature ecology & evolution* 2: 1258
533 Morales HE, Pavlova A, Joseph L, Sunnucks P. 2015. Positive and purifying selection in
534 mitochondrial genomes of a bird with mitonuclear discordance. *Molecular Ecology* 24:
535 2820-37
536 Muller H. *Biol. Symp.1942,* 6: 71-125.

537 Müller HJ. 1940. Bearing of the Drosophila work on systematics. In *The New Systematic*, ed. JS
538 Huxley, pp. 185-268: Clarendon Press, Oxford
539 Murrell B, Moola S, Mabona A, Weighill T, Sheward D, et al. 2013. FUBAR: A fast, unconstrained
540 bayesian AppRoximation for inferring selection. *Molecular Biology and Evolution* 30:
541 1196-205
542 Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Kosakovsky Pond SL. 2012. Detecting
543 individual sites subject to episodic diversifying selection. *PLoS Genetics* 8
544 Nadachowska-Brzyska K, Burri R, Olason PI, Kawakami T, Smeds L, Ellegren H. 2013.
545 Demographic divergence history of pied flycatcher and collared flycatcher inferred from
546 whole-genome re-sequencing data. *PLoS Genetics* 9: e1003942
547 Olson JR, Cooper SJ, Swanson DL, Braun MJ, Williams JB. 2010. The Relationship of Metabolic
548 Performance and Distribution in Black-Capped and Carolina Chickadees. *Physiological*
549 *and Biochemical Zoology* 83: 263-75
550 Pool JE. 2015. The mosaic ancestry of the Drosophila genetic reference panel and the D.
551 melanogaster reference genome reveals a network of epistatic fitness interactions.
552 *Molecular biology and evolution* 32: 3236-51
553 Presgraves DC. 2003. A fine-scale genetic analysis of hybrid incompatibilities in Drosophila.
554 *Genetics* 163: 955-72
555 Price TD, Bouvier MM. 2002. The evolution of F1 postzygotic incompatibilities in birds.
556 *Evolution* 56: 2083-89
557 Qvarnström A, Ålund M, McFarlane SE, Sirkiä PM. 2016. Climate adaptation and speciation:
558 particular focus on reproductive barriers in Ficedula flycatchers. *Evolutionary*
559 *applications* 9: 119-34
560 Qvarnström A, Rice AM, Ellegren H. 2010. Speciation in Ficedula flycatchers. *Philosophical*
561 *Transactions of the Royal Society B: Biological Sciences* 365: 1841-52
562 Qvarnström A, Wiley C, Svedin N, Vallin N. 2009. Life-history divergence facilitates regional
563 coexistence of competing Ficedula flycatchers. *Ecology* 90: 1948-57
564 R Core Team. 2017. R: A language and environment for statistical computing. *R Foundation for*
565 *Statistical Computing, Vienna, Austria. URL* http://www.R-project.org/.
566 Rand DM, Haney RA, Fry AJ. 2004. Cytonuclear coevolution: the genomics of cooperation.
567 *Trends in Ecology & Evolution* 19: 645-53
568 Rolfe D, Brown GC. 1997. Cellular energy utilization and molecular origin of standard metabolic
569 rate in mammals. *Physiological reviews* 77: 731-58

570 Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, et al. 2017. DnaSP 6:
571 DNA sequence polymorphism analysis of large data sets. *Molecular biology and evolution*
572 34: 3299-302
573 Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC. 2004. Effects of purifying and
574 adaptative selection on regional variation in human mtDNA. *Science* 303: 223-26
575 Ryman N, Jorde PE. 2001. Statistical power when testing for genetic differentiation. *Molecular*
576 *Ecology* 10: 2361-73
577 Ryman N, Palm S, André C, Carvalho GR, Dahlgren TG, et al. 2006. Power for detecting genetic
578 divergence : differences between statistical methods and marker loci. *Molecular Ecology*
579 15: 2031-45
580 Schumer M, Cui R, Powell DL, Dresner R, Rosenthal GG, Andolfatto P. 2014. High-resolution
581 mapping reveals hundreds of genetic incompatibilities in hybridizing fish species. *Elife*
582 3: e02535
583 Seehausen O, Butlin RK, Keller I, Wagner CE, Boughman JW, et al. 2014. Genomics and the
584 origin of species. *Nature Reviews Genetics* 15: 176
585 Sicard A, Kappel C, Josephs EB, Lee YW, Marona C, et al. 2015. Divergent sorting of a balanced
586 ancestral polymorphism underlies the establishment of gene-flow barriers in Capsella.
587 *Nature communications* 6: 7960
588 Sirkiä PM, McFarlane SE, Jones W, Wheatcroft D, Ålund M, et al. 2018. Climate-driven build-up
589 of temporal isolation within a recently formed avian hybrid zone. *Evolution* 72: 363-74
590 Smeitink JAM, Sengers RCA, Trijbels JMF. 2004. *Oxidative phosphorylation in health and disease*.
591 Kluwes academic / Plenum publishers.
592 Svedin N, Wiley C, Veen T, Gustafsson L, Qvarnström A. 2008. Natural and sexual selection
593 against hybrid flycatchers. *Proceedings of the Royal Society of London B: Biological*
594 *Sciences* 275: 735-44
595 Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA
596 polymorphism. *Genetics* 123: 585-95
597 Tieleman BI, Versteegh MA, Fries A, Helm B, Dingemanse NJ, et al. 2009. Genetic modulation of
598 energy metabolism in birds through mitochondrial function. *Proceedings of the Royal*
599 *Society of London B: Biological Sciences* 276: 1685-93
600 Vaidya G, Lohman DJ, Meier R. 2011. Cladistics multi-gene datasets with character set and
601 codon information. *Cladistics* 27: 171-80

602 Vallin N, Rice AM, Bailey RI, Husby A, Qvarnström A. 2012. Positive feedback between
603 ecological and reproductive character displacement in a young avian hybrid zone.
604 *Evolution* 66: 1167-79
605 van den Heuvel L, Smeitink J. 2001. The oxidative phosphorylation (OXPHOS) system: nuclear
606 genes and human genetic diseases. *Bioessays* 23: 518-25
607 Walsh B, Lynch M. 2018. *Evolution and selection of quantitative traits*. Oxford University Press.
608 Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. 2018. Datamonkey 2.0: A
609 modern web application for characterizing selective and other evolutionary processes.
610 *Molecular Biology and Evolution* 35: 773-77
611 Willett CS, Burton RS. 2001. Viability of Cytochrome C genotypes depends on cytoplasmic
612 backgrounds in Tigriopus californicus. *Evolution* 55: 1592-99
613 Yang Z. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. *Molecular Biology and*
614 *Evolution* 24: 1586-91

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616

617 **Abbreviations**:

618 mtDNA = mitochondrial DNA

619 nDNA = nuclear DNA

620 BDMI = Bateson Dobzhansky Muller incompatibilities

621 RMR = resting metabolic rate

622 OXPHOS = oxidative phosphorylation pathway

623 FLF* = Fu's and Li's F* statistic

624 FLD* = Fu's and Li's D* statistic

625 SNP = single nucleotide polymorphism

626

627 **Tables:**

Table 1. Genetic diversity and divergence

Pop = gene-population; Seg = segregating sites, (st) = singletons; H = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity; D_{XY} = average number of nucleotide substitutions per site between populations (with Jukes-Cantor adjustment); k = average number of nucleotide differences between populations; (SD) = standard deviation.

Pop	Seg (st)	H	Hd (SD)	π (SD)	D_{XY} (SD)	k
MT-collared	39 (13)	18	0.976 (0.020)	0.00148 (0.00020)	0.03302 (0.00244)	174.745
MT-pied	65 (55)	22	1.000 (0.014)	0.00162 (0.00015)		
Nuc-collared	311 (113)	19	1.000 (0.017)	0.00280 (0.00008)	0.00422 (0.00031)	107.125
Nuc-pied	254 (88)	19	1.000 (0.017)	0.00269 (0.00008)		

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629

Table 2. Neutrality tests per gene and per species

FLD* = Fu and Li's D* statistic; FLF* = Fu and Li's F statistic; Tajima's D = Tajima's D neutrality statistic. Red indicates negative and significant (* = P<0.5; ** = P<0.02; *** = P<0.001), yellow is negative, green is positive, black is zero (no variation).

An asterisk after the gene-name (for a mitochondrial gene) indicates only extracted data was available for that gene.

	Collared flycatchers			Pied flycatchers		
Gene	**FLD***	**FLF***	**Tajima's D**	**FLD***	**FLF***	**Tajima's D**
Complex I						
Mitochondrial genes						
12S	-3.9861**	-3.7507**	-1.6038	-2.9181*	-3.0771**	-1.9825*
16S	-4.8867**	-4.6199**	-1.9670*	-3.2516*	-3.3062**	-1.8781*
ND1	-1.4105	-1.4268	-0.8288	-1.8862	-2.02957	-1.3919
ND2	-0.7600	-0.5738	0.0085	-3.2898*	-3.4395**	-2.1575*
ND3	0.7833	0.8868	0.6837	-1.7805	-1.7956	-0.9906
ND4	-1.1539	-1.3518	-1.0953	-3.1051*	-2.9786*	-1.3157
ND4L	-2.9365*	-2.7376*	-1.1157	-1.7957	-2.0368	-1.6790
*ND5**	-0.6283	-0.5671	-0.1460	-0.8218	-1.1716	-1.5168
ND6	-1.8019	-1.9108	-1.2470	-2.6773*	-2.8955*	-2.0574*
Nuclear genes						
NDUFA2	0.0000	0.0000	-	0.0000	0.0000	-
NDUFA4	-1.5203	-1.6305	-1.1648	0.0000	0.0000	-
NDUFA5	-0.5736	-0.8252	-1.1200	0.0000	0.0000	-
NDUFA6	0.0000	0.0000	-	0.0000	0.0000	-
NDUFA8	-1.5203	-1.6305	-1.1648	0.0000	0.0000	-
NDUFA11	0.8750	0.6152	-0.3872	0.0000	0.0000	-
NDUFB1	-0.7823	-0.7997	-0.4626	0.0094	-0.0096	-0.0526

NDUFB3	-0.5736	-0.6302	-0.4849	-0.1052	-0.0231	0.2079
NDUFB6	0.0000	0.0000	-	0.0000	0.0000	-
NDUFB9	-1.5203	-1.6305	-1.1648	0.0000	0.0000	-
NDUFB10	0.0000	0.0000	-	0.6578	0.6794	0.4171
NDUFS1	1.3036	1.1186	0.1123	-2.2054	-2.3859	-1.7153
NDUFS4	-1.2251	-1.4688	-1.4224	0.6578	0.6794	0.4171
NDUFS5	0.0000	0.0000	-	0.0000	0.0000	-
NDUFS7	0.0000	0.0000	-	0.0000	0.0000	-
NDUFS8	0.2317	0.2508	0.1800	0.0000	0.0000	-
NDUFV1	0.0000	0.0000	-	0.0000	0.0000	-
NDUFV2	0.2317	0.4161	0.6868	-0.1432	-0.1610	-0.1294
Complex III						
Mitochondrial genes						
CytB	-3.3245**	-3.3030**	-1.8579*	-4.2346**	-4.3038**	-2.4301**
Nuclear genes						
UQCRQ	0.0000	0.0000	-	0.0000	0.0000	-
UQCRC2	-0.0279	-0.1423	-0.3585	-0.7259	-0.6050	-0.0146
UQCR10	0.0000	0.0000	-	0.0000	0.0000	-
UQCR11	0.0000	0.0000	-	0.0000	0.0000	-
Complex IV						
Mitochondrial genes						
CO1	-0.9560	-1.1432	-0.9752	-3.1892*	-3.1586*	-1.6174
*CO2**	-0.7312	-0.6848	-0.2363	-2.1748	-2.4100	-1.8566*
CO3	-1.6433	-1.6085	-0.7830	-4.1893**	-4.2057**	-2.2952**
Nuclear genes						
COX4I1	-0.5736	-0.4201	0.1991	-1.5203	-1.6305	-1.1648

COX6C2	0.0000	0.0000	-	0.6578	0.3850	-0.5622
COX7A	0.6578	0.3850	-0.5622	0.6578	0.6794	0.4171
COX7B	0.0000	0.0000	-	0.0000	0.0000	-
COX7C	0.0000	0.0000	-	0.0000	0.0000	-
Complex V						
Mitochondrial genes						
ATP6	-1.7066	-2.1458	-1.9573*	-4.7183**	-4.6629**	-2.3910**
*ATP8**	0.6578	0.6794	0.4171	0.0000	0.0000	-
Nuclear genes						
ATP5F1B	0.6578	0.3850	-0.5622	0.8750	1.1103	1.2249
ATP5F1C	0.8750	1.0353	0.9807	-0.5736	-0.4951	-0.0452
ATP5MF	0.0000	0.0000	-	0.0000	0.0000	-
ATP5MG	0.0000	0.0000	-	0.0000	0.0000	-
ATP5PB	0.0000	0.0000	-	0.0000	0.0000	-
ATP5PD	0.0000	0.0000	-	-2.0222	-2.1606	-1.5108
ATP5PF	0.6578	0.8832	1.0951	0.6578	0.7926	0.7938
Concatenated alignments						
All nuclear	-0.3591	-0.4120	-0.3404	-0.2638	-0.2290	-0.0391
All MT	-0.3045	-0.4738	-0.6294	-3.3702**	-3.4298**	-1.9422*

630

Table 3. Sites with positive selection

We used three programs to identify sites with positive selection, namely CodeML, MEME and FUBAR. A likelihood-ratio test (LRT) in CodeML calculated whether models with positive selection were more likely than models without positive selection. When this test was not significant, and positive selection was thus not more likely than no selection, the identified 'positive sites' were not accepted.

In the 'total' column, only sites are mentioned where the LRT indicates selection and that were identified by at least two methods (the models in CodeML count as a method each). BEB = Bayesian Empirical Bayes-method.

The genes for which there is no coding variation are not included in this analysis.

An asterisk after the gene-name (for a mitochondrial gene) indicates only extracted data was available for that gene.

Gene	**CodeML**			**MEME**	**FUBAR**	**Total**
	LRT	*BEB model 2*	*BEB model 8*			
Complex I						
Mitochondrial DNA						
ND1	Selection	20	20	20	20	20
ND2	No selection	X	X	30	X	No selection
ND3	No selection	X	X	X	X	No selection
ND4	Selection	4, 10, 14	4, 10, 14, 18	X	4	4, 10, 14
ND4L	No selection	X	X	X	X	No selection
*ND5**	Selection	13,350,577	13,350,577	34	X	13,350,577
ND6	No selection	X	X	X	X	No selection
Nuclear DNA						
NDUFA5	Selection	63	63	X	X	63
NDUFB3	Selection	8, 57, 99	8, 57, 99	X	57	8, 57, 99
NDUFS1	Selection	22, 529	22	X	X	22
NDUFS5	No selection	X	287	X	X	No selection
NDUFS7	Selection	21	21	X	21	21
NDUFV1	Selection	7	7	X	7	7
No coding variation: NDUFA2, NDUFA4, NDUFA6, NDUFA8, NDUFA11, NDUFB6, NDUFB9, NDUFB10, NDUFV2						
Complex III						
Mitochondrial DNA						
CytB	Selection	23, 31, 265	23, 31, 265	265	31, 265	23, 31, 265
Nuclear DNA						
UQCRC2	Selection	2, 188, 311, 341	2, 188, 311, 341	2	2, 188, 341	2, 188, 311, 341

No coding variation: UQCRQ, UQCR10, UQCR11						
Complex IV						
Mitochondrial DNA						
CO1	Selection	5, 27, 280	5, 27, 280	5	27	5, 27, 280
*CO2**	No selection	X	X	X	X	No selection
CO3	Selection	10, 12, 18	10, 12, 18	12	12	10, 12, 18
Nuclear DNA						
No coding variation: COX4I1, COX6C2, COX7A, COX7B, COX7C						
Complex V						
Mitochondrial DNA						
ATP6	No selection	5, 13	5, 7, 13	13	13, 46	No selection
*ATP8**	No selection	X	X	X	X	No selection
Nuclear DNA						
ATP5PF	Selection	30	30	X	X	30
No coding variation: *ATP5F1B, ATP5F1C, ATP5MF, ATP5MG, ATP5PB, ATP5PD*						

633 **Figure Captions:**

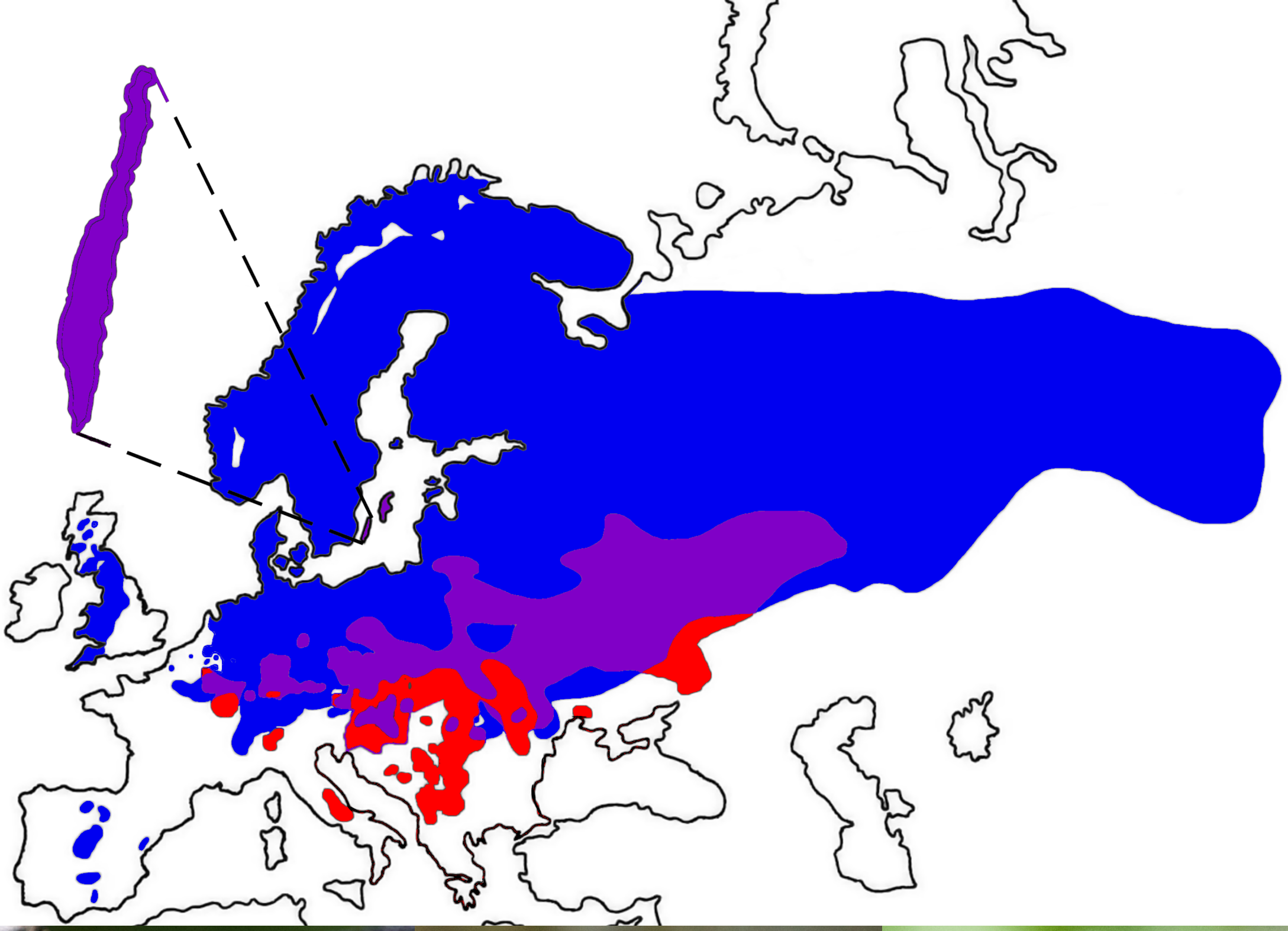
634 **Figure 1: A.** The distribution of *Ficedula* flycatchers in Europe. The distribution of pied
635 flycatchers is indicated by blue, whereas the distribution of the collared flycatchers is indicated
636 by red. The sympatric range is indicated in purple. Our study site is Öland, as enlarged in the
637 image. **B.** A male collared flycatcher (black with a white belly, a white collar, a white forehead
638 patch and white on its wings). **C.** A hybrid male (intermediate plumage; a broken white collar).
639 **D.** A male pied flycatcher (black or brown with a white belly and a small white forehead patch).

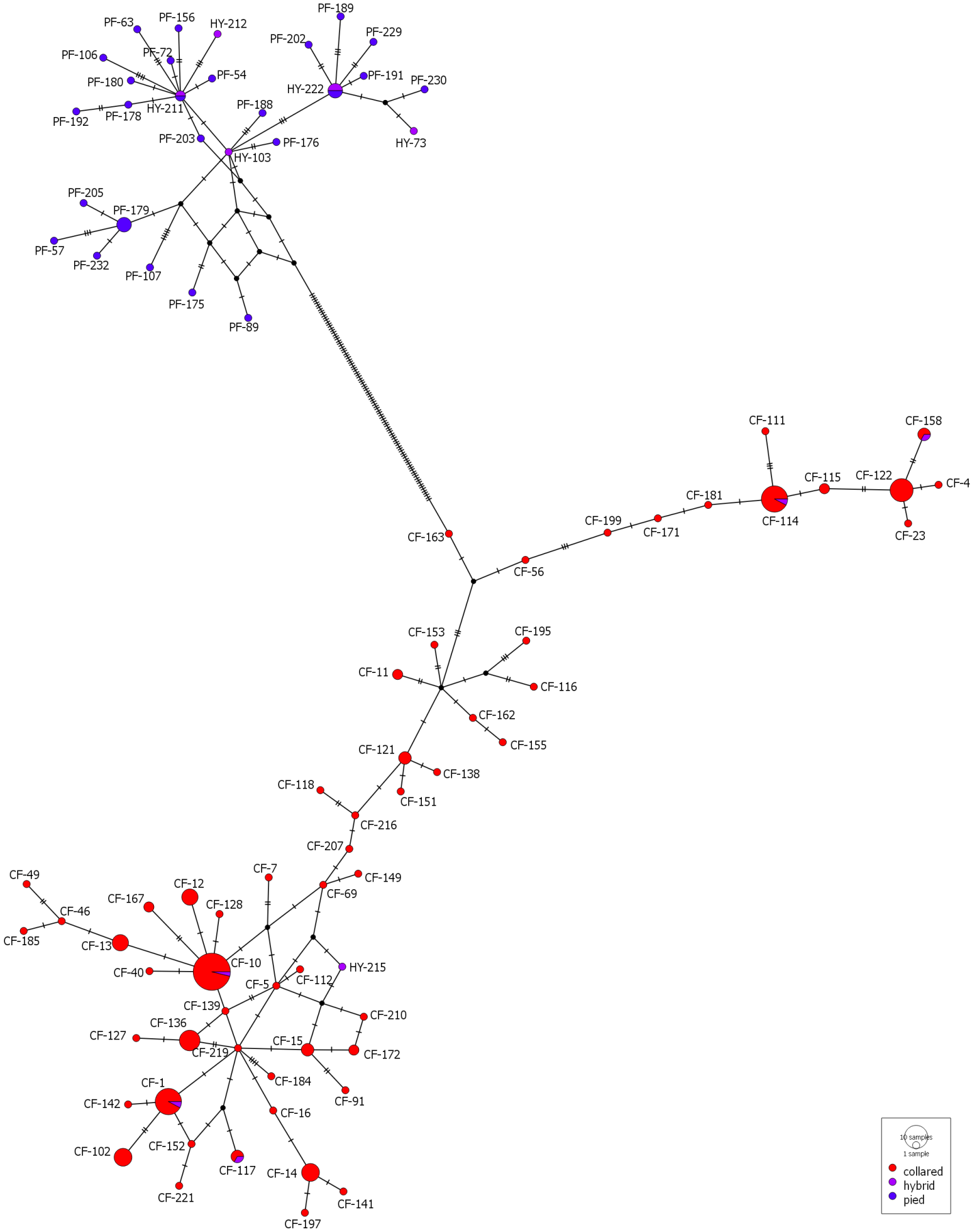
640

641 **Figure 2.** A haplotype network with all individuals based on concatenated alignment of the
642 genes ND1, ND2, ND3, ND4, ATP and 12S. Larger circles indicate several individuals have the
643 same haplotype. The reference sequence is present in the circle indicated by 'CF-10'.
644 Perpendicular bars on the edges indicate the number of differences that separates the two
645 haplotypes. Collared and pied flycatchers cluster together based on species.

646 **Figure 3.** A haplotype network with all individuals based on concatenated alignment of the
647 nuclear OXPHOS-related genes. No individuals share the same haplotype. Perpendicular bars
648 on the edges indicate the number of differences that separates the two haplotypes. Collared
649 and pied flycatchers cluster together based on species.

650 **Figure 4.** Manhattan plot with P-values of the Fisher's Exact test for all shared SNPs. The Y-axis
651 is the -log(P-value), and the X-axis corresponds to the complex each SNP is a part of. Black and
652 grey SNPs are located on the nDNA, whereas orange SNPs are located on the mtDNA. The blue
653 dashed line indicates P=0.05 and the red line indicates P=0.05/179 (P-value with Bonferroni
654 correction), all SNPs above the red line are significantly different between the two species after
655 accounting for multiple testing.

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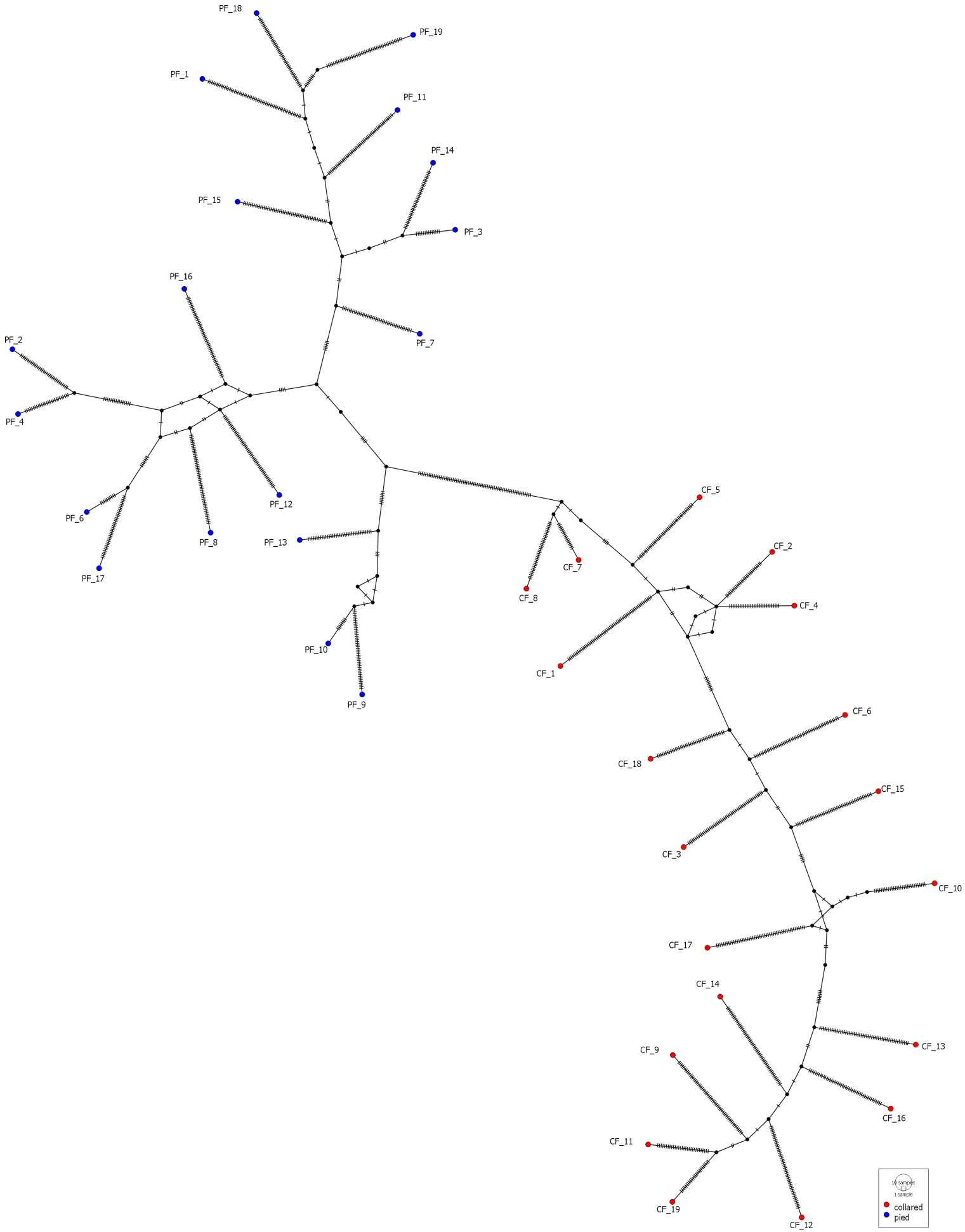
PF-156
HY-212
PF-189
PF-229
PF-202
PF-106
PF-72
PF-54
PF-191
PF-230
PF-180
HY-222
PF-188
PF-192
PF-178
HY-211
PF-203
PF-176
HY-73
HY-103
PF-205
PF-179
PF-57
PF-232
PF-107
PF-175
PF-89
CF-111
CF-158
CF-115
CF-122
CF-4
CF-181
CF-114
CF-199
CF-171
CF-23
CF-163
CF-56
CF-153
CF-195
CF-11
CF-116
CF-162
CF-155
CF-121
CF-138
CF-118
CF-151
CF-216
CF-207
CF-7
CF-149
CF-49
CF-12
CF-69
CF-167
CF-128
CF-46
CF-185
CF-13
CF-10
HY-215
CF-40
CF-112
CF-5
CF-139
CF-210
CF-136
CF-127
CF-15
CF-219
CF-172
CF-184
CF-1
CF-91
CF-142
CF-16
CF-102
CF-152
CF-117
CF-14
CF-221
CF-141
CF-197
10 samples
1 sample
collared
hybrid
pied


PF_18
PF_19
PF_1
PF_11
PF_14
PF_15
PF_3
PF_16
PF_7
PF_2
PF_4
PF_12
PF_6
PF_8
PF_13
PF_17
PF_10
PF_9
CF_5
CF_2
CF_7
CF_8
CF_4
CF_1
CF_6
CF_18
CF_15
CF_3
CF_10
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CF_16
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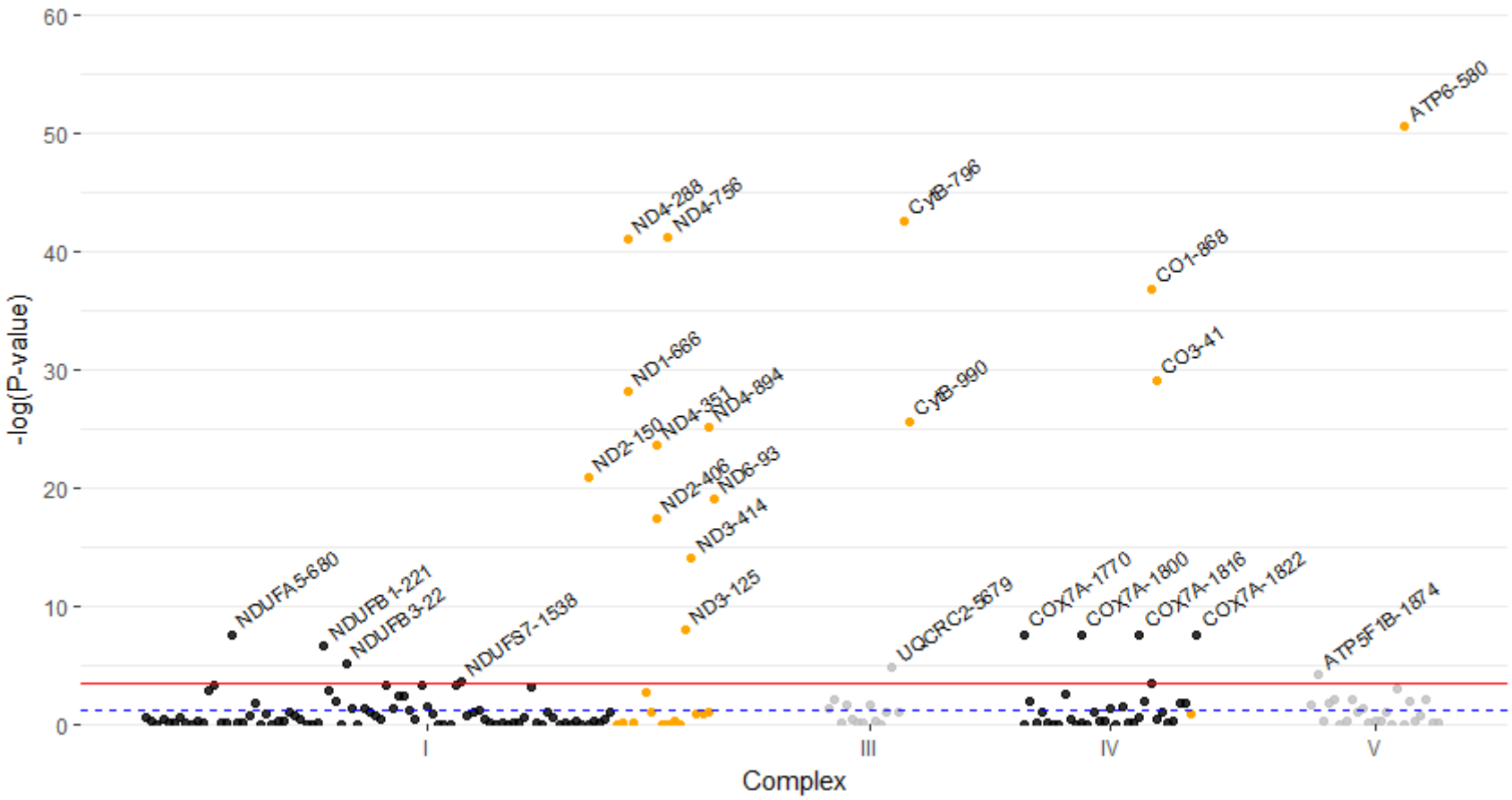
CF_19
CF_12
10 samples
1 sample
collared
pied


-log(P-value)
60
50
40
30
20
10
0
ATP6-580
ND4-288
ND4-756
CytB-796
CO1-868
ND1-666
CO3-41
ND4-351
ND4-894
CytB-990
ND2-150
ND2-406
ND6-93
ND3-414
NDUFA5-680
NDUFB1-221
NDUFB3-22
NDUFS7-1538
ND3-125
UQCRC2-5679
COX7A-1770
COX7A-1800
COX7A-1816
COX7A-1822
ATP5F1B-1874
I
III
IV
V
Complex