1   **First genetic evidence that invasive bullhead (*Cottus* L. 1758) in Scotland is of English**

2   **origin and the difficulty of resolving the European *Cottus* species taxonomy**

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25   **Abstract**

26   The European bullhead (*Cottus gobio*) is widely distributed across Europe and within the UK

27   is native to England and Wales, where it is protected under the Habitats Directive. In

28   Scotland, however, the species is considered invasive and thriving populations are recorded

29   in the Forth and Clyde river catchments, and the Ale Water in the Scottish Borders. The

30   genetic identity of the Scottish populations has not been established. There is also debate

31   about the status of the European bullhead and its validity as single species, a species

32   complex with several unresolved species, or distinct different species in its European

33   distribution range. There is therefore a need to determine the taxonomy and likely source of

34   the novel Scottish populations. Genetic analyses using cytochrome oxidase 1 (COI) mtDNA

35   sequences were undertaken on specimens from the Forth and Clyde catchments, and

36   combined with the results of morphological characteristics to provide a comprehensive

37   assessment of the taxonomic classification for Scottish bullheads. There was considerable

38   variation in morphological characteristics between populations within Scotland and a wider

39   range of variability than previously recorded for English populations. Genetically the Scottish

40   populations were very closely related to English specimens, supporting the hypothesis of

41   introduction directly from England to Scotland . In terms of broader relationships, Scottish

42   specimens are genetically more closely related to the ostensible species Chabot fluviatile

43   *Cottus perifretum*, which has been suggested as one of a complex of species across Europe.

44   Morphologically they exhibit characteristics on the spectrum between *C. perifretum* and *C.*

45   *gobio*. There is an urgent need for the clarification of the taxonomy of *Cottus* sp(p). to avoid

46   confusion in future publications, legislation and management practices relating to bullheads

47   throughout the UK and Europe.

50

51   **Significance Statement**

52   The European bullhead is native to England and afforded considerable protection under the

53   EC Habitats Directive, but considered invasive in Scotland where populations of unknown

54   origin have been recorded since the 1950's. A molecular and morphological examination of

55   bullhead from across Scotland confirmed that Scottish populations were a phylogenetic

56   match to the protected English species. Phylogenetic relationships of *Cottus* species are

57   complex and individuals from Scotland are genetically closer to the ostensible species *C.*

58   *perifretum,* while morphologically on the spectrum between *C. gobio* and *C. perifretum*.

59

60   1 INTRODUCTION

61   The genus *Cottus* (Cottidae, Scorpaeniformes) is widely distributed throughout Europe,

62   Siberia, North America and Asia, and represents the most speciose taxonomic group of

63   freshwater sculpins (Goto *et al*., 2015). In Europe, the European bullhead *Cottus gobio* L.

64   1758, has a continent-wide distribution, but the taxonomy of this species is under debate,

65   with some authors distinguishing a large number of species across Europe based on genetic

66   and morphological differentiation (Sonnenberg *et al.,* 2007; Freyhof *et al*., 2005; Kottelat,

67   1997).

68   Historically, morphological descriptors were used to distinguish between related similar

69   species. Variations in morphological characteristics both between and within populations of

70   *Cottus* across Europe have been well documented. Nybelin (1958) divided European *Cottus*

71   species into two groups based on the number of pores present on the chin. Those within the

72 *C. gobio* group possess one median chin pore whilst two pores are present in specimens

73 within the Alpine bullhead (*Cottus poecilopus* Heckel 1837) group. Koli (1969) examined a

74 selection of northern European bullheads to determine geographical variations in external

75 morphology. Three morphological features showed considerable variation: the distribution

76 and presence/absence of skin prickling, the number of pores found along the lateral line,

77 and the overall length of the lateral line. The density and distribution of skin prickling was

78 found to best distinguish bullheads from different regions, albeit with considerable variation

79 within populations. Prickling was most evident in specimens from northern Sweden, the

80 inland waters of Finland and the former eastern Soviet Union. Bullheads originating from

81 Britain, the majority of Sweden and the region extending from the Pyrenees to the

82 Carpathian mountains showed a lower density of prickling overall, with prickles completely

83 absent in some specimens. These results led Koli (1969) to propose the existence of two

84 morphological strains of bullheads that originated from separate post-glacial migratory

85 pathways, with a secondary zone of intergradation in regions where these pathways

86 historically overlap. Molecular studies have subsequently exposed an intergradation zone in

87 the Rhine that contains two distinct species, which also interbreed resulting in hybrids

88 (Englbrecht *et al.*, 2000).

89 Traditional morphological descriptors were unable to satisfactorily resolve the taxonomic

90 uncertainty present within the European bullhead species complex, but in combination with

91 molecular markers, a more detailed picture of European *Cottus* phylogeny has been

92 developed. The early examination of allozyme markers from different populations across

93 Europe provided evidence of heterozygosity that had not previously been observed. Studies

94 were conducted across south-western Germany and the connecting water bodies of

95 northern France (Riffel & Schreiber, 1995, 1998); north-east Bavaria (Hänfling & Brandl,

96   1998 a,b), and southern France (Eppe, 1999), and showed significant allozyme diversity

97   between populations of bullheads from the same drainage basin. Mitochondrial DNA

98   (mtDNA) and microsatellite loci have also been used to examine genetic relationships

99   between populations in Finland (Kontula & Väinölä, 2004) and the drainage waters of the

100 Adriatic (Šlechtová *et al.*, 2004). On a larger scale, a comprehensive phylogeographical

101 analysis across the majority of the *C. gobio* European range was conducted by Englbrecht *et*

102 *al.* (2000), based on 12 new microsatellite loci that were published in an earlier study

103 (Englbrecht *et al.*, 1999). Analysis of molecular distances between haplotypes of different

104 populations identified six distinct clades within the *C. gobio* species complex. The results

105 were consistent with the genetic variability within the Rhine basin documented in earlier

106 allozyme studies (Riffel & Schreiber 1995, 1998). Volckaert *et al.* (2002) identified a seventh

107 clade in the Brittany-Loire region of France and described a larger geographical range for the

108 North Sea and Lower Rhine clades than previously recognised. Subsequent studies have

109 been able to assign populations to these clades based on mtDNA from closely associated

110 populations (Knapen *et al.*, 2003) and microsatellite markers from populations distributed

111 across Europe (Hänfling *et al.*, 2002). Freyhof *et al.* (2005) proposed the existence of nine

112 genetic lineages, containing fifteen distinct species based on a combination of genetic,

113 morphometric and morphological characteristics of cottids from across Europe, and

114 proposed a single species, *Cottus perifretum* (Freyhof *et al.* 2005)*,* for Great Britain, but the

115 status of these species remains unclear.

116 On the British Isles, bullheads are widely distributed across their native range in England and

117 Wales (Boon & Lee, 2005; Carter *et al.*, 2004; Smyly, 1957; Tomlinson & Perrow, 2003;

118 Wheeler, 1977). In Scotland, an invasive designation is in place owing to a perceived threat

119 to native salmonids and other fishes, due to competition and displacement from niche

120   overlap (e.g. Carter *et al.*, 2004; Elliott, 2006; Pihlaja *et al.*, 1998), and bullhead consumption

121   of salmonid eggs (e.g. Gaudin & Caillere, 2000; Palm *et al.*, 2009; Smyly, 1957). Bullheads

122   were thought to have been introduced to Scotland in the 1950's (Maitland, 1972, 1977;

123   Smyly, 1957; Tomlinson & Perrow, 2003), although a lack of economic or angling value has

124   led some to suggest the introduction was accidental (Mills & Mann, 1983). Early reports of

125   bullhead in Scottish waters came from the Clyde catchment (Patton, 1951) with subsequent

126   reports from areas within the Forth catchment (Gemmel, 1962; Maitland, 1977; Morris,

127   1978). Three isolated populations were known in catchments in Scotland in 2001 (Adams &

128   Maitland, 2001), where the species is considered invasive, but since then they have

129   expanded to other areas. The waters draining into the Clyde and Forth estuaries and a small

130   area of the Tweed catchment and the Kirtle Water in the Annan catchment currently

131   constitute all known occurrences of bullheads in Scotland.

132   Differences in morphological characteristics exhibited by bullheads from across England

133   were noted by Wheeler (1977), who reported two distinct morphologies in eastern England;

134   a smooth skinned form with a complete lateral line extending across the length of the body

135   wall, and a second morph with spinulose (prickled) skin and an incomplete lateral line.

136   Freyhof *et al.* (2005) similarly found that samples from the River Wensum and Great Ouse in

137   Southeast England possessed the dense prickling typical of the proposed species *C.*

138   *perifretum*, but individuals examined from the River Wharfe in Yorkshire displayed an

139   elongated body shape and a decrease in the amount of prickling present under the pectoral

140   fin. The finding of two separate spawning tactics in bullheads from the north and south of

141   England by Fox (1978) appeared to support the idea of two separate genetic lineages on the

142   British Isles. Hänfling *et al.* (2002) examined microsatellite markers in specimens from a

143   range of locations across the range of *C. gobio*, and found a close association between

144   specimens from the north of England and Wales. A noticeable divergence was found when

145   comparing these specimens against those of southern England and the Scheldt. Significant

146   genetic variability is therefore evident between bullhead populations in southern England

147   and those found in northern England and Wales.

148   Freyhof *et al*. (2005) were limited in the material they were able to examine from Britain

149   and no examination was conducted on specimens of bullhead originating from the novel

150   populations that have established in Scotland. As the origin of Scottish bullhead populations

151   is unknown, further investigation into the morphological and molecular characteristics of

152   individuals from the northernmost limits of the British range will further our understanding

153   of the level of speciation present within the British Isles. Confirmation of taxonomic identity

154   is of particular importance owing to the emphasis placed on *C. gobio* conservation across

155   Europe and throughout England.

156   This study aimed to provide the first genetic evidence of the taxonomic identity of Scottish

157   bullheads as well as morphological analyses of bullheads from the Forth and Clyde

158   catchments. The results of these analyses were compared to the descriptions provided for a

159   range of species grouped under the *C. gobio* species complex. Results are discussed in the

160   context of taxonomic revisions within the genus *Cottus* and their contribution to our

161   understanding of bullhead's invasive designation in Scotland, which is of importance to

162   future management strategies.

163

164   2 MATERIALS AND METHODS

165   Specimens were collected from five sites on the east coast of Scotland and one location on

166   the west coast (Figure 1). Samples from the east coast covered four separate rivers (the

167   River North Esk, Burdiehouse Burn, Braid Burn and River Almond) and two locations on the

168   same river that were separated by impassable barriers (Brox Burn and River Almond). Fish

169   were obtained from routine sampling undertaken by the Forth Rivers Trust personnel for

170   fisheries management purposes in the summer of 2015, using a Smith-Root LR-24 backpack

171   electrofisher. They were euthanised on-site in a solution of 100mgL^{-1} benzocaine and

172   subsequently individually frozen at -20 °C.

173

174   2.1 Morphological Analysis

175   Fish were thawed at room temperature for 30 minutes in preparation for morphological

176   examination and subsequent tissue sampling for DNA extraction. Bullhead specimens from

177   the River Almond (n=33), River North Esk (n=35), Burdiehouse Burn (n=55), Braid Burn

178   (n=35), Brox Burn (n=34) and Earn Water (n=37) were examined for both quantitative and

179   descriptive morphological features. Morphological characters were selected based on their

180   capacity to discriminate between bullhead populations in earlier publications (Freyhof *et al.*,

181   2005; Koli, 1969; Riffel & Schreiber, 1998). These included meristic traits commonly

182   observed in fish studies and bullhead-specific factors concerning the distribution and

183   density of modified dermal skin prickles (Figure 2). The coverage and extent of prickling was

184   reported as a visual estimate of the percentage of the posterior body wall covered in

185   prickles, measured with each fish in a side-on orientation. When conducting fin ray counts,

186   rays extending from the same pterygiophore were counted as 1.5 rays, as in the work of

187   Freyhof *et al.* (2005). Body weight, standard length (from tip of the snout to end of hypural

188   complex) and full length (from tip of the snout to tip of caudal fin) were recorded for each

189   specimen. All examinations were completed with the assistance of an Olympus SZ51

190   dissection microscope (Olympus, Tokyo, Japan) using magnifications ranging from 8x - 40x**.**

8

191 Meristic traits were compared between each population. Statistical analysis was conducted

192 using Minitab 17 Statistical Software (2010). As assumptions for parametric analysis were

193 not met for meristic trait data, non-parametric analysis was undertaken using Kruskal-Wallis

194 testing with post-hoc Mann-Whitney U tests between selected variables using a Holm-

195 Bonferroni correction (Holm, 1979). A one-way ANCOVA was conducted to compare skin

196 prickling distribution with standard length, whilst also assessing any location variability.

197 Post-hoc analysis of location-driven differences in prickling density was conducted using a

198 Tukey's test.

199

200 2.2 DNA Isolation, PCR and Sequencing

201 Two specimens from each location (River Almond, River North Esk, Burdiehouse Burn, Braid

202 Burn, Brox Burn and Earn Water) were examined under an Olympus SZ51 dissection

203 microscope (Olympus, Tokyo, Japan) and the organs exposed using a cranio-caudal incision

204 from the gill covers to the vent region. Approximately 25mg of muscle and 10mg of liver

205 tissue were extracted from each individual and placed in individual sterile microcentrifuge

206 tubes. DNA was extracted using Qiagen® DNeasy Blood and Tissue kit (Qiagen, Hilden,

207 Germany), as per the protocol provided. A 30 minute RNA digestion step using 1µl RNAseA

208 (5µg/ml) was added to the end of the lysation process to ensure a purified end product. The

209 mitochondrial gene cytochrome c oxidase I (COI) was PCR amplified using the combination

210 of primers COI FW 5'-TTCTCGACTAATCACAAAGACATT- 3' and COI REV 5'-

211 TAGACTTCAGGGTGACCAAAGAATCA-3' (Sonnenberg *et al.*, 2007), and puReTaq Ready-to-go

212 PCR beads (GE Healthcare). The total reaction mix comprised 2.5µl DNA, 1µl COI FW primer,

213 1µl COI REV primer, 20.5 µl dH$_2$O and a PCR bead. PCR amplifications consisted of a 15

214 minute initial denaturation phase at 95°C; followed by 45 cycles of: 20 seconds at 94°C

215   (denaturing), 90 seconds at 52°C (annealing) and 90 seconds at 72°C (extension); and a final

216   extension phase at 72°C for 8 minutes. PCR products corresponding to the expected size

217   were then gel isolated using a 25 minute electrophoresis process, and extracted from the

218   gel using an Ultraclean® 15 DNA purification kit (Mo BIO, California, USA). The DNA content

219   of each purified product was measured using a NanoVue Plus Spectrophotometer (GE

220   Healthcare, Massachusetts, USA). A final sequencing mixture composed of 2μl of either COI

221   FW or COI REV primer, and 75ng/15μl DNA (with the discrepancy in volume made up with

222   dH$_2$O), was obtained. Forward and reverse reactions were sequenced by Eurofins Genomics

223   in Germany. COI gene sequences of the tissue samples are available in GenBank (accession

224   numbers MN218451 – MN218456).

225

226   2.3 Molecular Phylogenetic Analysis

227   The COI sequences were identified by BLAST analysis. Phylogenetic analysis was undertaken

228   using the six mtDNA COI sequences of Scottish bullhead amplified in this study and those of

229   32 other species within the genus *Cottus* published in Genbank. Sequences from Atlantic

230   salmon (*Salmo salar* L. 1758) and European ruffe (*Gymnocephalus cernua* (L. 1758)) were

231   added to the alignment as outgroup. Sequences were aligned using Geneious version7

232   (http://www.geneious.com, Kearse *et al*., 2012), with visual editing to confirm placement

233   accuracy. An alignment of 475 base pairs was obtained after all gaps had been excluded. The

234   alignment was analysed with Maximum Likelihood (ML) and Bayesian methods.

235   ML and bootstrap analyses were undertaken using the programme PhyML (Guindon &

236   Gascuel, 2003; Guindon *et al*., 2010) with the Tamura-Nei (TN) model of nucleotide

237   substitutions (Posada & Crandall, 1998) and a γ -distribution with a fixed proportion of

238   invariable sites and a transition/transversion ratio (Ti/Tv) estimated from each data set (40-

239   taxon alignment: six rate categories, γ = 0.156). The computation was completed under the

240   TN93+G+F substitution model, using Akaike Information Criterion (AIC).

241   Bayesian analysis was performed using MrBayes 3.2.6 (CIPRES Science Gateway V. 3.3)

242   (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The program was set to

243   operate with a GTR substitution model, γ - distribution and four Monte Carlo Markov chains

244   (MCMC; default temperature = 0.2). A total of 10,000,000 generations were calculated

245   based on trees sampled every 100 generations, with a prior burn-in of 25,000 generations

246   (2,500 sampled trees were discarded). The 75,000 post-burn-in trees obtained were used to

247   construct a majority-rule consensus tree, where reported posterior probabilities correspond

248   to the frequency at which a given node occurred in a post-burn-in tree.

249   A pair-wise distance calculation based on Kimura's two-parameter model (Kimura, 1980)

250   was performed on the same alignment, using MEGA 7.0 (Kumar *et al.*, 2016).

251

252   2.4 Multivariate analysis of morphological characteristics and genetic distance

253   To investigate the combined relationship between genetic distance and fish morphology,

254   data were analysed in a multivariate framework using non-metric multidimensional scaling.

255   Matrices of each of the morphology and genetic distance were created based on species

256   and study locations and the first two axes compared. All statistical analysis was undertaken

257   using the R programming environment (R Core Team, 2019) using the packages *cluster*

258   (Maechler, et al., 2019), *ggplot2* (Wickham, 2016), *ggrepel* (Slowikowski, 2019), *MASS*

259   (Venables & Ripley, 2002) and, *vegan* (Oksanen, et al., 2019).

260

261   3. RESULTS

262   3.1 Morphological and Meristic Analyses

263   In terms of general morphology, an oval-shaped dorsal fin, rounded pectoral fins and four

264   pelvic fin rays were present on all bullhead. A membrane attaching the first and second

265   dorsal fin and a second one connecting the caudal dorsal fin with the body wall were also

266   present in all specimens, regardless of their origin. All examined fish showed dense skin

267   prickling around and inferior to the pectoral fin, although the coverage of prickles across the

268   body wall varied greatly between specimens of all sizes. The results of ANCOVA showed that

269   the extent of prickling did not vary consistently with length ($F = 0.07$; df = 1, 218; $p = 0.795$),

270   but did vary between sites ($F = 11.63$, df = 5, 218; $p < 0.001$) (Figure 3). Based on Tukey post-

271   hoc testing no obvious geographical patterns in the significant differences between sample

272   locations were observed.

273   There was a substantial amount of intraspecific variation in all examined fin ray counts

274   (Figure 4). Significant differences in second dorsal fin ray counts (Kruskal-Wallis test; $H =$

275   21.69, df = 5, $p < 0.05$), anal fin ray counts ($H = 22.03$, df = 5, $p < 0.05$) and pectoral fin ray

276   counts ($H = 67.11$, df = 5, $p < 0.05$) were found between populations (Figure 4b, c and d).

277   Values obtained were compared with those published for native English bullheads (Maitland

278   & Campbell, 1992; Kottelat & Freyhof, 2007). Results showed a broader variation in pectoral

279   fin rays counts, anal fin ray counts and second dorsal fin ray counts than has been previously

280   described for bullheads in Britain (Figure 4b, c and d).

281   Bullheads sampled from the Earn Water had a significantly higher average number of

282   pectoral fin rays than bullheads from all other locations (Mann-Whitney U test; Figure 5).

283   Significant differences in pectoral fin ray counts were also found when comparing the River

284   Almond and Burdiehouse Burn (Mann-Whitney U test, Figure 5). Anal fin ray counts differed

285   when comparing the River Almond with the Braid Burn and the River Earn; and the Brox

286   Burn with the Braid Burn and the River Earn (Mann-Whitney U test Figure 5). Significant

287   differences were also found when comparing the second dorsal fin ray counts of the River

288   Almond samples against those of the River North Esk, the Burdiehouse Burn and the Braid

289   Burn (Mann-Whitney U test, Figure 5).

290

291   3.2 Molecular Phylogenetic Analysis

292   Analyses of the six Scottish bullhead COI sequences obtained in this study, along with the

293   COI sequences of *Cottus* from across North America, Asia and Europe, showed separation in

294   the *Cottus* genus based on geographic origin of the species (Figure 6). ML bootstrap support

295   (MLBS) for some deeper branches of the phylogenetic tree was fairly low (less than 50%),

296   although higher support was found with Bayesian posterior probabilities (BPP). The weakly

297   supported deep branches in these phylogenetic trees suggest that the separation between

298   members of the *Cottus* genus is not well defined here.

299   The European *Cottus* sequences analysed in this study formed a well-supported clade in

300   both analyses (0.99 BPP, 85% MLBS). Three distinct clades are visible within the western

301   European *Cottus* group, along with the separation of four distinct sequences that do not

302   cluster well with any of the other groupings, or each other. Scottish bullhead sequences

303   formed a clade with the sequence from an English population classified on GenBank as *C.*

304   *perifretum*, by Sonnenberg et al. (2007), which is well supported by Bayesian analysis (0.98

305   BPP) and moderately supported by ML analysis (73% MLBS). The direct sister clade is

306   comprised of sequences from Chabot des Pyrénées (*C. hispaniolensis* (Bacescu & Bacescu-

307   Mester 1964)) and Chabot d'Auvergne (*C. duranii* (Freyhof *et al.* 2005)), of northern Spain

308   and France respectively. Sequences of *Cottus gobio* and Chabot du Béarn (*C. aturi* (Freyhof

309   *et al.* 2005)) formed a third clade as sister to the former two. Sequences of Chabot de

310   Rhénanie (*C. rhenanus* (Freyhof *et al.* 2005)), Chabot de l'Hérault (*C. rondeleti* (Freyhof *et al.*

311   2005))*, C. scaturigo* (Freyhof *et al*., 2005) and *C. microstomus* Heckel 1837 come off the

312   same node independently, and do not cluster with any of the other European *Cottus*

313   sequences. The overall support based on ML analysis for these clusters is relatively low

314   (Figure 6). The three aforementioned clades were fully supported in the Bayesian analysis.

315

316   A pair-wise distance calculation performed on 38 *Cottus* COI mtDNA sequences showed an

317   extremely high degree of similarity between the sequences of Scottish and English bullhead.

318   No differentiation was found amongst the British sequences except the Brox Burn, which

319   was marginally distinct, yielding a 0.21% divergence from the other British sequences (Table

320   1). Comparisons between the Scottish bullhead sequences derived from this study and all

321   other available sequences from *Cottus* species showed a higher divergence. However, low

322   interspecific divergence was shown across Europe (0.42-1.50%). All sequences originating

323   from specimens previously grouped as *C. gobio* yielded interspecific divergence values of

324   1.50 % or lower. Low values within similar thresholds were also found between European

325   cottids and Siberian sculpin (*C. sibiricus* Kessler 1889), originating from Siberia, and between

326   *C. sibiricus* and Spoonhead sculpin (*C. ricei* (Nelson 1876)) of Alaskan/north-west American

327   origin. *Cottus* sequences originating from North America species showed substantially

328   higher divergence compared to the sequences derived in this study (ranging from 1.72-

329   10.65%).

330

331   3.3 Multivariate analysis of morphological characteristics and genetic distance

332   Multivariate comparison of morphological data revealed a clustering of the Scottish

333   populations in a space between *C. gobio* and *C. perifretum* (Figure 7a). The morphology of

334   the Earn Water specimens was closer to *C. perifretum*, while the morphologies of the

335   Almond, North Esk and Braid Burn populations were closer to *C. gobio*.

336   Multivariate comparison of genetic distance data revealed tight clustering of our *Cottus*

337   species from multiple Scottish populations with *C. perifretum* (Figure 7b).

338   Furthermore, a Mantel test examining the direct correlation between the morphological and

339   genetic distance matrices revealed a significant relationship (r=0.505, p=0.001) indicating

340   that the morphological measurements collected were a good indication of genetic distance.

341

342   4. DISCUSSION

343   A combined approach of morphological and molecular markers is considered optimal when

344   examining the taxonomic identity of any species. One of the reasons is the long-standing

345   belief that miss-inferences can occur when gene sequence results of for example mtDNA are

346   studied in isolation (e.g. Hurst & Jiggins, 2005; Rognon & Guyomard, 2003). Combined

347   morphological and molecular markers have been applied in other studies of European

348   bullhead to discriminate between populations, hybrids and species (Freyhof *et al*., 2005;

349   Kontula & Väinölä, 2004; Riffel & Schreiber, 1995, 1998). Here we show that both the

350   morphological and genetic approach have highlighted similarities between Scottish and

351   English populations and emphasized the variability in both aspects across the broader

352   European context. Therefore, in the following discussion, we will use the names that have

353   been proposed for the species in the *Cottus* complex following Freyhof *et al*. (2005), but

354   treat them as ostensible given the ongoing debate over their taxonomic status.

355   The examination of morphological traits in Scottish bullhead uncovered a greater variation

356   in meristic factors than had previously been described (Freyhof *et al*., 2005; Kottelat &

357   Freyhof, 2007; Maitland & Campbell, 1992). When data from each of the six sampling

358 locations were compared, significant differences were found between populations in

359 median ray counts from the second dorsal fin, pectoral fin and anal fin, although the ranges

360 overlapped. Even though the differences were very subtle, results were significant with

361 differences of one ray in median counts (e.g. for pectoral fin ray counts in the Earn water

362 (median value of 14) compared to all other sites (13). Across these analyses a single location

363 could not be differentiated for all three of the morphological traits, nor was any found to be

364 uniquely different from the other Scottish populations. The River Almond and Brox Burn

365 were the only two locations sampled in this study that belong to the same river system.

366 Counts of anal fin rays proved to be similar for both locations, and distinctly different from

367 the Braid Burn and Earn Water populations. As bullheads are a relatively sedentary and

368 territorial species (Tomlinson & Perrow, 2003), mixing between populations is restricted to

369 high flow, downstream flooding events or rare density-mediated migrations. Due to these

370 restrictions to mixing events, populations in the same river system can exhibit

371 morphological differentiation. The morphological variation encountered in the studied

372 specimens is considered a result of natural variation and can be attributed to the higher

373 quantity of specimens examined in this study compared to the small numbers available in

374 reference material (e.g. Freyhof *et al.*, 2005). Adaptive radiation may be a contributing

375 factor, due to the limited migratory capacity of the species and lack of genetic mixing, but is

376 unlikely given the species was only introduced to Scotland in the 1950's.

377 Skin prickling has proven a useful measure of identifying different bullhead populations in

378 Northern Europe (Koli, 1969; Kontula & Väinölä, 2004), but was applied with less success in

379 Eastern Europe (Oliva & Hensel, 1962). Kottelat & Freyhof (2007) described dense prickling

380 in juvenile *C. perifretum*, but stated that prickling is greatly reduced or absent in mature

381 males. Koli (1969) also noted that larger specimens possess less prickles than smaller

382   individuals due to resorption of spines after the onset of maturity. There was no obvious

383   influence of age on prickling density in Scottish specimens, based on the relationship

384   between standard body length (as a proxy for age) and prickling coverage, although there

385   were pronounced differences between sites in the extent of prickling. There was no clear

386   geographical pattern in the significant differences found between sample locations.

387   Bullheads from the River Earn are the most geographically isolated population examined,

388   but significant differences in prickling density were only found between the River Earn and

389   one other location (the Brox Burn). Given prickling density is thought to decrease with age

390   (Koli, 1969) the lack of relationship between body length and prickling coverage was

391   unexpected, but could suggest that standard body length is a poor proxy for age in

392   bullheads and may be dependent on other environmental factors (e.g. Abdoli *et al*., 2007).

393   Thus, age-dependant prickling coverage may not have been examined fully in this analysis.

394   All bullheads examined in this study had some degree of skin prickling and a complete

395   lateral line. This is contrary to the smooth-skinned form with a complete lateral line, and

396   prickled-skin form with incomplete lateral line described in England by Wheeler (1977).

397   However, the results are in agreement with the descriptions for British bullhead provided in

398   other studies (Freyhof *et al*., 2005; Kottelat & Freyhof, 2007; Maitland & Campbell, 1992).

399   Of the morphological traits examined, the presence of dermal prickling, complete lateral

400   line and presence of a membrane connecting the anal fin to the body wall were attributes

401   found in Scottish bullhead that have been previously assigned to *C. perifretum* (Freyhof *et*

402   *al*., 2005; Kottelat & Freyhof, 2007). This classic form of identification is a practical non-

403   invasive approach, but size restrictions require additional methods of identification to be

404   applied to adult specimens.

405   A molecular approach was therefore utilised in conjunction with morphological analysis.

406   Phylogenetic analyses of mtDNA sequences obtained from Scottish bullhead, in conjunction

407   with those reported for other *Cottus* species, provided evidence for three distinct clades in

408   European freshwater systems. In addition, four more sequences from European *Cottus* were

409   closely related, but not strongly associated with any of these clades. The combined results

410   of Englbrecht *et al*. (2000) and Volckaert *et al.* (2002) revealed seven clades in Europe. The

411   description of species ranges and cladistics given in Freyhof *et al*. (2005) supported six of

412   these clades, including a clade containing *C. perifretum*, confirmed by specimens from both

413   Great Britain and continental Europe. The results of the present study verify the presence of

414   a clade containing *C. gobio* (clade I of Englbrecht *et al*., 2000); one containing *C. duranii*

415   (clade VII of Volkaert *et al*., 2002), and a clade containing *C. perifretum* (clade IV of Volkaert

416   *et al*., 2002). In addition, the results of this study grouped *C. hispaniolensis* with *C. duranii,*

417   and *C. aturi* with *C. gobio*. The clade consisting of *C. gobio* and *C. aturi* was not well

418   supported (59% MLBS, 0.76 BPP). Englbrecht *et al*. (2000) and Freyhof *et al*. (2005)

419   positioned *C. aturi* into a different clade, and the low ML and Bayesian support in the

420   present study suggests that an alternative position in the phylogenetic tree is possible. The

421   clade consisting of *C. hispaniolensis* and *C. duranii* was moderately well supported (70% ML

422   and 1.00 BPP), and would suggest that the two species are very closely related. *Cottus*

423   *scaturigo*, *C. microstomus*, *C. rhenanus* and *C. rondeleti* were all found among the European

424   *Cottus* species, but did not form a strong association with any particular clade. Šlechtová *et*

425   *al*. (2004) were unable to discriminate between *C. rondeleti* and other closely associated

426   *Cottus* species using mtDNA, but a morphological description that described the species *C.*

427   *rondeleti* was given by Freyhof et al. (2005). Similarly, Šlechtová *et al*. (2004) and Englbrecht

428   *et al*. (2000) were unable to distinguish *C. scaturigo* from neighbouring *C. gobio* using

429   molecular methods, but Freyhof *et al*. (2005) used morphological evidence to describe the

430   species *C. scaturigo*.

431   The ML tree produced using bootstrap analysis showed that with the exception of *C.*

432   *poecilopus*, all European cottids grouped closely together. *Cottus poecilopus* was

433   distinguished from other European *Cottus* species by Koli (1969), who noted morphological

434   differences in chin pore arrangement (one pore is found in all species grouped under the *C.*

435   *gobio* species complex and two pores are found on *C. poecilopus*). The genetic dissimilarity

436   of *C. poecilopus* from other European *Cottus* species reflected the morphological

437   differences. Bayesian support for the divergence of the European cottids was high (0.99

438   BPP), as was the support for the British clade containing the sequence of *C. perifretum* and

439   the Scottish bullhead samples of this study (0.98 BPP). Bootstrap values were somewhat

440   lower, giving moderate support at 85% MLBS and 73% MLBS, respectively. Hillis & Bull

441   (1993) found that bootstrap values ≥70% correspond to at least a 95% probability that the

442   simulated clade obtained is real. The combined results of Bayesian and ML analysis are

443   therefore deemed complimentary. This is further verified by the results of pairwise distance

444   calculations conducted across all *Cottus* sequences. Five of the six Scottish bullhead

445   sequences and the sequence provided for *C. perifretum* showed no divergence when

446   compared against each other, and the sixth sequence showed only 0.21% divergence to the

447   other Scottish bullhead samples. Interestingly, there was also no divergence found when

448   comparing the two European sequences of *C. hispaniolensis* and *C. duranii*; and the

449   collective grouping of *C. microstomus*, *C. rhenanus* and *C. scaturigo,* despite each being

450   considered an independent species. The ML tree produced in this study showed that the

451   sequences within these two groupings were found within the same clade, with very little

452   distinction between sequences. A high degree of genetic similarity is therefore expected,

453 and the determination of species-level distinctions between these populations has been the

454 subject of other, studies (e.g. Freyhof *et al*., 2005). Although the comparison between the

455 newly sequenced Scottish bullheads and the sequence published as *C. perifretum* has

456 produced a similar result showing minimal divergence, nothing in the analyses suggest that

457 any of the compared British sequences differ on a molecular level. Whilst mtDNA typically

458 evolves faster than single-copy nuclear DNA (Avise, 2000), mtDNA genes are integral to the

459 speciation process (Gershoni *et al*., 2009; Lane, 2009) and have been described as an

460 unambiguous measure of species identity in a recent publication (Hill, 2016). COX1

461 genotypes have been a fundamental component of phylogenetic studies for decades and

462 are highly effective at defining species boundaries (Hill, 2016). The results obtained from

463 COI mtDNA analysis of Scottish bullhead therefore demonstrate that Scottish bullheads are

464 genetically identical to cottids from England*,* representing an isolated branch of the same

465 species. The published sequence for the ostensible species *C. perifretum* used for

466 comparison in the present study originated from England (Sonnenberg *et al.,* 2007). All six

467 sequences (four from Belgium and two from England) available on GenBank from that study

468 are 100% identical and therefore in agreement with the southern England and Belgium

469 group described by Hänfling *et al*. (2002). The similarity between this sequence and those

470 obtained from Scottish bullheads does not support the differentiation inferred for northern

471 and southern English bullheads, or British bullheads and those from Belgium, that were

472 discussed in Hänfling *et al*. (2002) based on microsatellite loci.

473

474 The combined morphological and molecular approach utilised in this study has provided a

475 more reliable means of identification. This method has shown that Scottish and English

476 bullhead are genetically equivalent, thereby confirming the presence of the native English

477 bullhead*,* in Scotland. The proposed reclassification of *Cottus gobio* as a species complex

478 and subsequent description of at least 15 ostensible *Cottus* species (summarised in Freyhof

479 *et al.* 2005) is still under debate. Originally two *Cottus* species had been recognized in

480 Europe, *C. gobio* and *C. poecilopus* (Nybelin, 1958). Molecular data are available for only ten

481 of these 15 proposed species. According to Ward (2009), when COI sequence divergence is

482 below 0.5% there is a very high probability (>95%) that sequences stem from conspecific

483 fish. This drops to a probability of 70% for a divergence of up to 1% and 45% for a

484 divergence of up to 1.5% (Ward, 2009). Therefore, based on our sequence data (Table 1),

485 the *Cottus* specimens from this study as well as the ostensible European species *C. duranii*,

486 *C. hispaniolensis*, *C. microstomus*, *C. perifretum*, *C. rondeleti*, *C. rhenanus, C. scaturigo,* with

487 the potential exception of *C. aturi*, should be referred to as *C. gobio*, as the sequence

488 divergence shows values between 0.0% and 1.5% and an average divergence of 0.53%.

489 Using the morphological and genetic information in a multivariate framework has shown

490 significant consensus in the genetic sequences of the Scottish populations with that of *C.*

491 *perifretum*, but more variability in the population morphologies. Furthermore, the high

492 degree of correlation between the genetic and morphological data suggests, that even using

493 a subset of morphological characters (meristic data) provides a good agreement between

494 the two measures of species determination.

495

496 There is undoubtedly substantial variation at the molecular and morphological level for

497 *Cottus gobio* across its distribution in Europe. There is an urgent need for a coordinated

498 multidisciplinary approach across the European countries, to finally settle the taxonomic

499 debate and to enable targeted management and legislative approaches as well as avoiding

500 publications under wrong species names. For this, we need not only standard morphological

501 and single genetic markers, but mensural characters, skeletal structures, relevant ecological

502 information, and multiple genetic markers statistically analysed to confidently separate

503 existing populations into potentially different species.

504

505 The results of this study have shown for the first time that a single *Cottus* species resides in

506 Great Britain. The native species range spans England and Wales, with sites in Scotland

507 where this species has been introduced. Current UK legislation pertaining to bullhead in

508 Great Britain lists *C. gobio* as the native species. *Cottus gobio* is protected under Annex II of

509 the EC Habitats Directive due to the considerable decline of bullheads throughout some

510 regions in Europe (Knaepkens *et al.*, 2005). Eleven locations in England are currently

511 designated Special Areas of Conservation (SACs) for bullhead (Boon & Lee, 2005) as a result

512 of the Habitat's Directive implementation in the UK. These areas receive enhanced

513 protection measures, with the purpose of protecting the vulnerable species recognised in

514 the area. This has resulted in the protection of bullhead in regions where populations are

515 thriving. Population levels in England have commonly been considered robust (Wheeler,

516 1977; Mills & Mann, 1983; Copp, 1992;), leading Carter *et al*. (2004) to suggest an

517 exemption of bullhead from the requirements of the Habitats Directive in the UK. This

518 practice has already been undertaken in Finland, where bullhead (assumed *C. gobio*) is an

519 introduced non-native species (Pihlaja *et al*., 1998; Carter *et al*., 2004).

520 The EC Habitats Directive components of UK law will likely be revisited when the UK departs

521 from the European Union, and would provide a window of opportunity to update legislation

522 concerning bullheads in the UK. We therefore suggest that the legislation be updated to

523 include the term "species complex" with reference to *C. gobio* enabling future proofing of

524 the legislation to accommodate future research outcomes. In recently conducted risk

525   screenings for translocated freshwater species, Dodd *et al.* (2019) classified *Cottus gobio* as

526   a species with medium invasive potential in Scotland. Scottish bullhead populations stem

527   from genetic material that originated in England. The Scottish freshwater communities in

528   which they are found are of a similar composition to those in England and the presence of

529   bullheads within Scottish rivers is consequentially thought to elicit similar ecological

530   interactions. It is therefore suggested that Scottish bullhead be classified similarly to native

531   English bullhead. UK legislation needs to be updated to reflect the relative robustness of

532   populations that exist throughout the UK.

533

539

540   CONTRIBUTIONS

541   J.M. - ideas, data generation, data analysis, manuscript preparation.

542   R.B. - data analysis, manuscript preparation.

543   J.D. – data analysis, manuscript preparation.

544   S.R. - ideas, data analysis, manuscript preparation, funding.

545

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