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*Integrated genetic and morphological data support eco-evolutionary divergence of Angolan and South African populations of *Diplodus hottentotus**

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1 **Integrated genetic and morphological data support eco-evolutionary divergence of**
2 **Angolan and South African populations of *Diplodus hottentotus*.**

3
4 **Running headline:** Eco-evolutionary divergence in *Diplodus* spp.

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19 Gwilliam MP & Winkler AC contributed equally to this manuscript

ABSTRACT

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24 The *Diplodus* genus presents multiple cases of taxonomic conjecture. Among these the *D.*
25 *cervinus* complex was previously described as comprising three subspecies that are now
26 regarded as separate species: *D. cervinus*, *D. hottentotus* and *D. omanensis*. *Diplodus*
27 *hottentotus* exhibits a clear break in its distribution around the Benguela Current system,
28 prompting speculation that Angolan and South African populations flanking this area may be
29 isolated and warrant formal taxonomic distinction. This study reports the first integrated
30 genetic (mtDNA and nuclear microsatellite) and morphological (morphometric, meristic and
31 colouration) study to assess patterns of divergence between populations in the two regions.
32 High levels of cytonuclear divergence between the populations support a prolonged period of
33 genetic isolation, with the sharing of only one mtDNA halotype (12 haplotypes were fully
34 sorted between regions) attributed to retention of ancestral polymorphism. Fish from the two
35 regions were significantly differentiated at a number of morphometric (69.5%) and meristic
36 (46%) characters. In addition, Angolan and South African fish exhibited reciprocally
37 diagnostic colouration patterns that were more similar to Mediterranean and Indian Ocean
38 congeners, respectively. Based on the congruent genetic and phenotypic diversity we suggest
39 that the use of '*hottentotus*', whether for full species or subspecies status, should be restricted
40 to South African *D. "cervinus"* to reflect their status as a distinct 'species- like unit', while
41 the relationship between Angolan and Atlantic/Mediterranean *D. "cervinus"* will require
42 further demo-genetic analysis. This study highlights the utility of integrated genetic and
43 morphological approaches to assess taxonomic diversity within the biogeographically
44 dynamic Benguela Current region.

45 **Key words:** taxonomy; fish; morphometric; meristic; mitochondrial; microsatellite

INTRODUCTION

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Within the family Sparidae there are 35 genera and 118 species described (Hanel & Tsigenopoulos, 2011). The genus *Diplodus* comprises 12 species, for which a number of sub-species have been described based on geographical differences and, often subtle, morphological variation (Hanel & Tsigenopoulos, 2011). While there is a general consensus relating to the taxonomy within the genus, Heemstra & Heemstra (2004) have suggested that many sub-species described around the Benguela Current system, a prominent marine biogeographic barrier, should be raised to full species status.

The *Diplodus cervinus* complex was previously described as comprising three subspecies: *Diplodus cervinus cervinus* (Mediterranean Sea and northeastern Atlantic Ocean), *D. c. hottentotus* (around southern Africa from Angola to Mozambique) and *D. c. omanensis* (Indian Ocean, endemic to Oman – see Figure I), but these taxa are now regarded as separate species (*D. cervinus*, *D. hottentotus* (Heemstra & Heemstra, 2004) and *D. omanensis* (Bauchot & Bianchi, 1984)). *Diplodus hottentotus* has a distinct break in its distribution, with no records of this species along the Namibian or South African west coast. It has been suggested that the southern Angolan and South African populations of *D. hottentotus* flanking this distribution break may be isolated by the cold water marine biogeographic barrier formed by the Benguela Current (Floeter *et al.*, 2008). Several studies have been conducted on the life history of *D. cervinus* from the Canary Islands (Pajuelo *et al.*, 2003a & b), Algeria (Derbal & Kara, 2006; 2010), South Africa (Mann & Buxton 1992, 1987, 1998),

70 and Angola (Winkler *et al.*, 2014 a,b). While there are significant differences between the life
71 history parameters of the northern Atlantic & Mediterranean populations and the Angolan &
72 South African populations, this could be due to sampling biases and the use of suspect aging
73 and sexual pattern determination techniques. Moreover, there have been no taxonomic
74 comparisons among Atlantic populations. As the Benguela Current system has been
75 implicated as a major biogeographic barrier to gene flow and to be driving population-, sub-
76 species-, and species-level divergences among marine fish in the region, empirical analysis of
77 the eco-evolutionary relationship between Angolan and South African *D. hottentotus* is
78 required.

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81 The objective of this work was to explore the possible divergence between hitherto
82 described conspecific Angolan and South African *D. hottentotus* populations. DNA barcoding
83 using mitochondrial DNA (mtDNA) cytochrome oxidase I sequences (Hebert *et al.*, 2003)
84 has been shown to be successful at identifying cryptic diversity among marine and freshwater
85 taxa (Nwani *et al.*, 2011; Pereira *et al.*, 2013). However, inferences based on COI, or any
86 single locus, may misrepresent a specie's/population's evolutionary history (Dupuis *et al.*
87 2012) and so genotyping of nuclear microsatellite loci was also performed here. As units
88 identified through genetic patterns can be supported by divergence in morphological or
89 biological traits (Thomas *et al.*, 2014) we also assess morphometric and meristic variation
90 between populations from the two regions. Both genetic and morphological data reveal high
91 levels of divergence between regional populations, which are interpreted along with other
92 information for *D. cervinus* and *D. omanensis* in a taxonomic context.

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MATERIALS & METHODS

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96 GENETIC ANALYSIS

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99 *Sampling and DNA extraction*

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102 A total of 168 individuals of *D. hottentotus* were collected from thirteen sampling
103 sites in Angola and South Africa, plus two outgroup individuals of *D. cervinus* from Turkey
104 (see Figure 2 & Supplementary Table I). Samples were obtained from a mixture of
105 recreational angling, spearfishing and local fish markets. A fin clip was removed from each
106 individual and preserved in 95% ethanol. Total genomic DNA was extracted following the
107 phenol-chloroform method described by Sambrook *et al.*, (1989) and visualised on a 1%
108 agarose gel.

109

110 *mtDNA sequencing and analysis*

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112

113 A 501bp fragment of the mtDNA cytochrome oxidase I (COI) gene was amplified
114 using PCR with unpublished species-specific primers DCCOIF (5'
115 TCATTCCGAGCCGAACTAAGC 3') and DCCOIR (5' TCCTGCAGGGTCAAAGAAAG
116 3'). PCRs comprised of 10 µl of BIOMIX (BioLine), 1.0 pMol of primer (both forward and

117 reverse), 6 µl of template DNA and 2 µl of sterile distilled water giving a total reaction
118 volume of 20µl. All PCRs were performed using the following reaction conditions: 120 s at
119 95°C, then 40 cycles of 30 s at 94°C, 30 s at 50°C, 60 s at 72°C, with a final extension step of
120 120 s at 72°C. PCR amplicons were cleaned using SureClean (BioLine) and sequenced in
121 both directions using Big Dye technology on an ABI 3730 DNA analyser (Applied
122 Biosystems®). Sequence chromatograms were examined and edited in CHROMAS
123 (Technelysium Ltd) and aligned using CLUSTAL W executed in BIOEDIT (Hall, 1999).
124 Genetic variation was described using haplotype diversity (h , Nei and Tajima, 1981) with
125 differentiation among samples quantified by Φ_{ST} (with significance assessed by 10 000
126 permutations) using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). A median joining network
127 was constructed in NETWORK (www.fluxus-engineering.com/sharenet.htm).

128

129 *Microsatellite DNA genotyping and analysis*

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132 Following testing of 18 published nuclear microsatellite sparid loci a subset of seven
133 polymorphic loci [DsaMS16, DsaM27, DsaMS34 (Perez et al., 2008), Dvul4, Dvul33,
134 Dvul58, Dvul84 (Roques et al., 2007a,b)] which provided consistent PCR products were
135 used to assess nuclear genetic variation among two samples from South Africa (Tsitsikamma
136 and Port Elizabeth) and one sample from Angola (Flamingo). Loci were individually
137 amplified by PCR using thermoprofiles consisting of 300s at 95°C, then 30 cycles of 30s at
138 92°C, 30s at a 55°C (but 50°C for Dvul33) and 30s at 72°C, and a final extension step of
139 72°C for 120s. All reactions used the following reaction mix: 5 µl of BIOMIX (BioLine), 0.5
140 pMol of primer (both forward and reverse), 3 µl of template DNA and 1 µl of sterile distilled

141 water giving a total reaction volume of 10 μ l. Alleles were separated using an AB3730 DNA
142 analyser and allele identity inferred using Peak Scanner 2.

143

144

145 Numbers of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), and
146 expected heterozygosity (H_E), were calculated using FSTAT 2.9.3.2 (Goudet, 1995).
147 Genotype frequency conformance at individual loci to Hardy-Weinberg equilibrium (HWE)
148 expectations and genotypic linkage equilibrium between pairs of loci were tested using exact
149 with default parameters in GENEPOP 3.3 (Raymond & Rousset, 1995). Multilocus values of
150 significance for HWE tests were obtained using Fisher's method (Sokal and Rohlf, 1995) to
151 combine probabilities of exact tests. The assumption of selective neutrality of the
152 microsatellite loci was tested using the outlier method implemented in LOSITAN (Antao et
153 al. 2008) following McKeown *et al.*, (2017). Genetic structuring without any prior
154 information was investigated using the Bayesian clustering method implemented in
155 STRUCTURE 2.3.4 (Pritchard et al. 2000). Briefly, the analysis identifies the most probable
156 number of genetically distinct groups (K) represented by the data and estimates assignment
157 probabilities (Q) for each individual (specifically their genomic components) to these groups.
158 Each MCMC run consisted of a burn in of 10^6 steps followed by 5×10^6 steps. Three
159 replicates were conducted for each K to assess consistency. The K value best fitting the data
160 set was estimated by the log probability of data [Pr(X/K)]. Clustering among individuals was
161 also assessed using Discriminant Analysis of Principal Components (DAPC) implemented in
162 ADEGENT (Jombart et al., 2010). Genetic differentiation among samples was also quantified
163 by single- and multi-locus values of the unbiased F_{ST} estimator, θ (Weir and Cockerham,
164 1984), calculated using FSTAT, with the significance of estimates tested by 10 000

165 permutations of genotypes among samples (Goudet et al., 1996). F_{ST} values were also
166 calculated employing the correction for potential null allele effects using FreeNA (Chapuis &
167 Estoup, 2007)

168

169 MORPHOLOGICAL ANALYSIS

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171

172 *Sample collection, preservation and analysis*

173 Fish were collected using spear fishing, hook-and-line, or purchased from local fish
174 markets from Benguela, Lucira, Namibe, Flamingo Lodge and Tombua in southern Angola
175 (n=25) and from Port Alfred, Port Elizabeth and Cape St Francis in South Africa (n=47).
176 After capture, fish were sacrificed and immediately placed in 10% formalin. After at least
177 one month, fish were transferred from the formalin to a 10% ethanol solution for three days, a
178 50% ethanol solution for three days, and final storage in a 70% ethanol solution.

179

180

181 Following preservation a total of 15 meristic counts and 47 morphometric
182 measurements were made on each fish following Hubbs & Larger (1947) and Richardson
183 (2011) and outlined in Supplementary Table II. All morphometric measurements were made
184 using digital callipers to the nearest 0.01 mm. If a fish was damaged and a particular
185 measurement was not possible, the measurement was estimated from a linear regression of

186 the form: $FL_i = mx_i + c$ where FL_i is the fork length of the damaged individual, m is the slope of
187 the model and x_i is the missing character and c is the y intercept.

188

189

190 Since morphometric data are continuous and the meristic data are discrete, statistical
191 analyses of both types were performed separately. Extreme outliers in the morphometric data
192 from each region were defined as those greater than three times the inter-quartile range,
193 below or above the first and third quartiles, and detected using a box plot analysis (Simon et
194 al., 2010). Significant correlations between size (FL) and morphometric characters may
195 accentuate such size differences (Simon et al., 2010) and complicate the morphometric
196 comparisons. To eliminate this common problem associated with allometric growth variation,
197 all morphometric measurements were size-adjusted to an overall mean fork length of 206.09
198 mm (the mean size of all samples) using the equation : $Y'_{ij} = \log Y_{ij} - b_j(\log FL_i - \log FL$
199 (overall)) (Reimchen et al., 1985, Senar et al., 1994, Simon et al., 2010).

200 Differences between size-adjusted morphometric and meristic character means
201 between Angolan and South African fish were tested using a two sample *t*-test. Both data sets
202 were then analysed using a multi-dimensional scaling (MDS) incorporating the Bray-Curtis
203 similarity measure. The extent of similarity between sites was assessed using a one-way
204 analysis of similarity (ANOSIM) using the statistical package PAST Version 2.16 (Hammer
205 et al., 2001) and were considered significant at $p < 0.05$.

206

207

RESULTS

208

209 GENETIC DIVERSITY

210 Pruning of mtDNA sequences permitted comparison of 501 sites across 96 individuals
211 (Angola n = 33; South Africa n = 40; Turkey n = 23 [two sequences obtained here and 21
212 from GenBank]) and revealed a total of 13 haplotypes. Haplotype diversity was higher in the
213 Angolan than South African sample (h (SD) = 0.73(0.06) and 0.36 (0.09) respectively) with
214 an intermediate value for Turkey (h (SD) = 0.58 (0.088)). There was a clear phylogeographic
215 partitioning of haplotypes between Angola and South Africa (Figure II) with only one
216 haplotype (Haplotype 7) shared between these regions. Three haplotypes were identified
217 among the Turkish samples and these were found to occupy central positions in the haplotype
218 network with one (Haplotype 6) being the most common haplotype among South African
219 samples, and the other two (Haplotypes 2 and 3) being the most common among the Angolan
220 samples (Figure II). The clear partitioning of haplotypes between Angola and South Africa
221 translated into large and highly significant Φ_{ST} (0.5; $P < 0.0001$). The Turkey sample also
222 displayed significant Φ_{ST} values against Angola and South Africa, but with much lower
223 values against Angola (0.06; $P < 0.05$) than South Africa (0.5; $P < 0.001$).

224

225

226 Information on microsatellite genetic variation for each sample / locus combination is
227 provided in Supplementary Table III. There were no significant deviations from random
228 associations of genotypes (linkage disequilibrium) detected for any pair of loci, either across
229 all samples (data pooled) or in any single sample, indicating that all loci assort independently.
230 No loci were identified as significant, putative non-neutral, outliers. All loci were variable in
231 each sample with the total number of alleles per locus ranging from two (DsaMS27) to 28
232 (Dvul84) with an average of 8.43. Although levels of variability differed across loci, multi-

233 locus variability indices were similar across all samples. Significant deviations from HWE
234 were found in 9 out of 21 locus / sample comparisons (Flamingo - 3 of 7 tests; Port Elizabeth
235 - 3 of 7 tests; Tsitsikamma - 3 of 7 tests), in eight cases due to heterozygote deficits, whilst
236 the Tsitsikamma / DsaMS34 comparison exhibited a heterozygosity excess. Bayesian
237 clustering unanimously supported a model of $K = 2$ ($P = 1$ for $K = 2$, and zero for other
238 models) with high assignment probabilities of all Flamingo (Angola) individuals to one
239 cluster and Tsitsikamma and Port Elizabeth (South Africa) individuals to the other cluster
240 (Figure III). This pattern was also evident following DAPC (Figure III). The pattern of
241 genetic structuring between Angolan and South African samples was also supported by
242 highly significant ($P < 0.0001$) pairwise F_{ST} values > 0.23 for comparisons between regions
243 with similar values obtained after correction for null alleles. No significant differentiation
244 was detected between Tsitsikamma and Port Elizabeth (F_{ST} without null allele correction =
245 0.019; with null allele correction = 0.017).

246

247 MORPHOLOGY

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250 Only one individual from the morphometric dataset in the Angolan samples was
251 identified as an extreme outlier and excluded from the subsequent analysis. The R^2 values for
252 the linear regressions were all above 0.6 before transformation. These were however all
253 below 0.05 after transformation, indicating that the transformed characters were free from a
254 size bias. 32 of the 46 morphometric measurements were significantly different between
255 South African and Angolan fish (Supplementary Table IV). The relationship between the
256 most significant morphometric characters and fork length further provides evidence for

257 separation between the two regions (Figure IV). Seven of the 15 meristic counts also revealed
258 significant differentiation between South African and Angolan fish (Supplementary Table V).
259 The MDS ordination plot for both morphometric and meristic characters separated South
260 African and Angolan individuals, with marginal overlap (Figure V). The ANOSIM results
261 suggested a similar result to the MDS but also verified that the groupings were significantly
262 different from one another ($P < 0.05$).

263

264

DISCUSSION

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266

267 Combined analysis of genetic and morphological variation can provide synergistic
268 insights into eco-evolutionary forces shaping biodiversity, as well as tools for conservation
269 and management (Carriera et al., 2017). The present study represents the first integrated
270 genetic and morphology based investigation within the *Diplodus* genus. A focus of this study
271 was to assess evidence for divergence between conspecific populations of *D. hottentotus* in
272 Angolan and South African waters. In line with *a priori* predictions, based on observations in
273 other coastal fish species of evolutionary independence of populations across the Benguela
274 Current system (Henriques, 2012; Henriques *et al.*, 2012; Henriques *et al.*, 2014; Henriques
275 *et al.*, 2016), high levels of genetic and morphological divergence between *D. hottentotus*
276 populations in the two regions were found, which should prompt discussion of taxonomic
277 revision in this species.

278

279

280 Congruent mtDNA and nuclear differentiation was observed between Angolan and
281 South African samples of *D. hottentotus*, with a lack of differentiation within regions (though
282 this could only be tested in South African waters). The mtDNA haplotype network, though
283 shallow and with only five nucleotide differences between maximally diverged haplotypes,
284 exhibited a clear phylogeographic structure: of 13 haplotypes resolved among South African
285 and Angolan samples only one (haplotype 7, a tip haplotype) was found in both regions. This
286 translated into high Φ_{ST} values between regions. Nuclear microsatellite variation also revealed
287 a high level of differentiation between Angolan and South African samples which was
288 supported by genetic clustering analyses. The strong assignment of individuals to their
289 ‘regional’ clusters provided no evidence of migrants or first generation hybrids between
290 regions. The cytonuclear differentiation between Angolan and South African samples
291 therefore clearly supports the hypothesis of restricted gene flow and absence of dispersal
292 across the Benguela Current

293

294

295 When applied to taxonomic questions genetic methods can avoid many of the pitfalls
296 of assessments based only on morphology, but traditional mtDNA-based approaches have
297 been criticised due to their over-reliance on strict exclusivity criteria such as reciprocal
298 monophyly or barcoding gaps (reviewed in Sites & Marshall 2004; Hudson & Coyne 2002;
299 Hudson & Turelli 2003; Moritz & Ciero, 2004). Specifically, mtDNA-based taxonomic
300 inferences applying such strict criteria may be compromised by specimen misidentification,
301 hybridisation and/or recent divergence (with the retention of ancestral polymorphism and
302 incomplete lineage sorting). In the present study genetic and phenotypic alignment for all
303 individuals excludes specimen misidentification, while patterns of nuclear differentiation
304 provide no support for hybridisation or any recent introgression including male-biased gene

305 flow. In light of this, the sharing of haplotype 7 between Angolan and South African samples
306 can be attributed to retention of ancestral polymorphism / incomplete lineage sorting. Even
307 more compelling evidence of retention of ancestral polymorphism is provided by the
308 presence of haplotype 6 (a central haplotype) in both the South African and Turkish samples
309 but its absence from Angolan samples, and conversely the sharing of haplotypes 2 and 3
310 between Turkey and Angola but their absence from South Africa. Collectively the genetic
311 patterns indicate considerable genetic divergence between Angolan and South African *D.*
312 *hottentotus* but that insufficient time has passed for mtDNA variation to be completely sorted.

313

314 All three haplotypes identified in the Mediterranean are shared with, and are the
315 common haplotypes among the African samples (two with Angola and one with South
316 Africa). This pattern contrasts with results from a similar mtDNA analysis of other *Diplodus*
317 species by Henriques (2012), who reported reciprocal monophyly of NE Atlantic *D. sargus*
318 (formerly *D. sargus sargus*) and African *D. capensis* (formerly *D. sargus capensis*) with an
319 estimated coalescence time of approximately 1.8 Ma. Similarly, Henriques (2012) reported a
320 higher degree of mtDNA divergence between Angolan and South African samples of *D.*
321 *capensis* than observed here for *D. hottentotus*. Coalescent depths among groups may vary
322 considerably due to differences in population size, mutation rate and time since speciation
323 (Monaghan *et al.*, 2009; Fujita *et al.*, 2012). Additionally, the faster generation time of *D.*
324 *capensis* / *D. sargus* (sexual maturity at 1.8 years: Richardson *et al.*, 2011) compared to *D.*
325 *hottentotus* / *D. cervinus* (sexual maturity at 4.9: Mann & Buxton, 1997) would permit faster
326 lineage sorting in *D. capensis* / *D. sargus* in a given time even if other mutation/demographic
327 processes were similar.

328

329

330 A high degree of phenotypic divergence between Angolan and South African *D.*
331 *hottentotus* was observed in morphometric ($R = 0.30$; significantly different mean values for
332 69% of characters) and meristic characters ($R = 0.42$; significantly different mean values for
333 46.1% of characters), and overall differentiation in the MDS ordination plots. Similar levels
334 of morphometric ($R = 0.34$) and meristic ($R = 0.35$) variation were reported between *D.*
335 *capensis* from Angola and South Africa (Richardson, 2011) however, despite the
336 aforementioned greater levels of genetic divergence fewer character means were
337 differentiated between both regions in that case. This indicates varying levels of plasticity /
338 adaptation and / or conservatism among these *Diplodus* species, which could compromise
339 taxonomic investigations based solely on phenotype. Plasticity and adaptation are also likely
340 to be key factors governing responses to future environmental change (King *et al.*, 2017).

341

342

343 Although general phenotype characteristics such as colouration are typically regarded
344 as highly plastic and of limited use as diagnostic characters, in the present study they do
345 reveal some intriguing macrogeographical patterns. As depicted in Figure I, Angolan
346 individuals were bronze in colour and lacked ventral abdominal stripes while those from
347 South Africa were more silver with intermittent belly stripes. Overall the Angolan colour
348 patterns were more similar to Mediterranean fish, while South African colour patterns were
349 more similar to fish from Oman. These phenotypic colouration patterns readily align with
350 those described previously by Bauchot & Bianchi (1984).

351

352

353 The genetic differences among South African and Angolan samples are compatible with a
354 prolonged period of population isolation and distinct evolutionary trajectories (Waples,
355 2008). The genetic diversity also aligns readily with regional differences in general
356 phenotype and morphology. Such congruent genetic-morphological divergence has driven
357 taxonomic reappraisals in other groups (e.g. Gobidae; Lima-filho *et al.*, 2016). With regard to
358 the use of ‘hottentotus’, whether for full species or subspecies status, this should be restricted
359 to South African *Diplodus “cervinus”* to reflect their status as distinct ‘species- like units’
360 (*sensu* Collins & Cruickshank 2013). Such a redefinition can be made conveniently due to the
361 clear geographical separation of both units. The relationship between Angolan and
362 Atlantic/Mediterranean *D. cervinus* will need to be further investigated through more
363 extensive phenotypic and genetic sampling. The present study highlights that DNA barcoding
364 has great value as an exploratory technique in taxonomy and for revealing cryptic diversity.
365 However, it also shows that this potential can only be maximised if traditional COI-based
366 approaches are complemented with data from other (independent) genetic loci, ontogenetic
367 data and an appreciation of the limit of applying strict thresholds/exclusivity criteria. In light
368 of the dynamics of speciation in the Benguela Current region, failure to do so or reliance on
369 one method may compromise species delimitation and an underestimation of coastal African
370 ichthyodiversity, thereby curtailing efforts to conserve evolutionarily distinct taxa in this
371 complex marine system.

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