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

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

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

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ABSTRACT

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

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

INTRODUCTION

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),

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

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

MATERIALS & METHODS

GENETIC ANALYSIS

Sampling and DNA extraction

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

mtDNA sequencing and analysis

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

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

Microsatellite DNA genotyping and analysis

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

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

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

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

MORPHOLOGICAL ANALYSIS

Sample collection, preservation and analysis

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

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

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

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

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

RESULTS

GENETIC DIVERSITY

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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