

1 **African freshwater eel species (*Anguilla* spp.) identification**
2 **through DNA barcoding**

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23 **Running header:** African freshwater eel DNA barcoding

24

25 **ABSTRACT**

26 Freshwater eels (*Anguilla spp.*) have a long and complex catadromous life cycle. This unique
27 feature, coupled with difficulty in separating species based on morphology, makes them
28 complex targets for conservation. In this study, we evaluate the utility of DNA barcoding using
29 cytochrome oxidase I (COI) to delimit the four species of African eels found in the Western
30 Indian Ocean region. We collected 75 individual fin clips from the four eel species (*A.*
31 *mossambica*, n = 51; *A. marmorata*, n = 17; *A. bengalensis*, n = 6; *A. bicolor*, n = 2) in the
32 rivers of KwaZulu-Natal, South Africa during 2016 - 2018. Phylogenetic analysis of the COI
33 sequences recovered all four species as monophyletic. Barcoding gap analyses were performed
34 and found that there was no overlap in inter- and intraspecific genetic distances. Consequently,
35 the use of COI-barcoding as an identification tool was found to be reliable for identifying
36 African eels to the species level, which suggests that this marker should be included in future
37 environmental DNA or metabarcoding studies.

38

39 **KEYWORDS**

40 cytochrome oxidase I, barcode gap analysis, *Anguilla*, Western Indian Ocean region, species
41 identification

42

43 **INTRODUCTION**

44 In the Western Indian Ocean region (WIO), Anguillid eels (*Anguilla spp.*) are
45 ecologically important species, yet the four species found in the region remain poorly
46 studied. Lack of reliable data has led to the species being poorly protected, with
47 populations potentially highly threatened or over exploited (Skelton 2001; Jacoby *et al.*
48 2015). This is similar to temperate eel species, which have also declined to the point of
49 endangerment (Lecomte-Finiger 2003; Castonguay and Durif 2015). African eels face a

50 multitude of growing threats. These include loss of habitat availability and connectivity,
51 changes to river flow and quality (Wasserman *et al.* 2011) as well as a growing harvest
52 demand, with Madagascar and Mauritius having recently entered the global eel trade
53 (Kaifu *et al.* 2019). African eels are the only long-distance catadromous species in the
54 WIO region, making them particularly vulnerable to changes in river connectivity
55 (Hanzen *et al.* 2019), but also making them good ecological indicators at the catchment
56 scale. Unfortunately, there is already evidence of their decline from the Réunion Island
57 where habitat loss, degradation of water quality as well as increased parasitic load have
58 negatively impacted on their abundance and recruitment (Valade *et al.* 2018). Réunion is
59 currently the only country in the region where a conservation plan for the four African
60 species has been implemented (Valade *et al.* 2018). But sustainable conservation will only
61 be possible through coordination with all countries within the distribution of the four
62 African species, and for most countries in the region data on eels is lacking.

63 Southern Africa's four species of freshwater eel (*Anguilla mossambica*, *A. bicolor*,
64 *A. bengalensis*, and *A. marmorata*; Skelton 2001) occur sympatrically (Jespersen 1942;
65 Jubb 1961; Robinet *et al.* 2007, 2008). These species exhibit complex catadromous
66 migratory patterns and undertake facultative catchment scale migration, which makes the
67 conservation planning, management and protection of these species difficult. After
68 spawning in the Indian Ocean in the Mascarene Plateau (Pous *et al.* 2009), pelagic larvae
69 proceed through several developmental stages into glass eels (sub-juveniles) before
70 migrating into southern African river systems. In freshwater systems, they develop into
71 elvers (juveniles) followed by the resident yellow eel stage (sub-adult form). Following
72 the progression to silver eels (adult or mature form), individuals will leave the freshwater
73 river systems and return to their marine spawning grounds off the coast of Madagascar to
74 breed and die.

75 Eels are a difficult group to identify morphologically (Watanabe 2004; Aoyama
76 2009), and in particular issues have been encountered separating the African species based
77 solely on morphology (Balon 1975). Species identification at the yellow and silver eel
78 stage of the life cycle is traditionally done using the Ege (1939) identification key. Species
79 identification at the larval (cylindrical and leaf-shaped leptocephalus larvae) and glass eel
80 stages are more complicated as this often requires the sacrifice of specimens, which
81 constrains recruitment monitoring and requires extensive taxonomic knowledge
82 (Réveillac *et al.* 2009).

83 Recent studies have highlighted the use of molecular techniques and data in
84 delimiting eel species (Gagnaire *et al.* 2007; Takeuchi *et al.* 2019). A variety of molecular
85 markers have been used to delimit species (Rhodopsin: Rahman *et al.* 2015; ATP6:
86 Takeuchi *et al.* 2019; 16SrRNA: Réveillac *et al.* 2009), but relatively few have used the
87 DNA barcode marker, cytochrome oxidase I (COI) (Rahman *et al.* 2015; Muchlism *et al.*
88 2017). DNA barcoding and the use of the 658-base pair (bp) protein-coding region of the
89 mitochondrial COI as standard animal DNA barcode is now well established as a
90 technique for species identification and discovery across a wide taxonomic variety of
91 species (Hebert *et al.* 2003; Radulovici *et al.* 2010). DNA barcoding relies on short,
92 standardized nucleotide sequences (DNA barcodes) as internal species tags, and rapid
93 species identification is facilitated by searchable online sequence repositories, such as the
94 Barcode of Life Data Systems (BOLD, www.barcodeoflife.org). Recent advances in next-
95 generation sequencing, metabarcoding and eDNA (environmental DNA) encourage the
96 use of standardized molecular markers in the field of DNA barcoding. In this study, we
97 evaluated the utility of COI to delimit the four species of southern African freshwater eel.
98 The reliability of the COI-barcode as an identification tool was also tested using DNA
99 barcode gap analyses. The DNA barcode gap is the difference between the greatest intra-

100 specific (within species) genetic distance and the smallest inter-specific (between species)
101 distance. Overlap between intra- and inter-specific genetic distances reduces the
102 reliability of COI barcoding.

103 **METHODS**

104
105 Animal ethics clearance for this study was obtained from the University of KwaZulu-
106 Natal Animal Ethics Committee (AREC/012/017D). We sampled eels using standard non-
107 invasive active and passive techniques, including the use of fyke nets and electrofishing in
108 rivers and impoundments across the KwaZulu-Natal Province (South Africa) during 2016 -
109 2018. We immediately anaesthetized captured eels using standard techniques (2-
110 phenoxyethanol at 0.5 ml/l) (Neiffer and Stamper 2009). Individuals were then weighed,
111 measured, and photographed. They were identified to the species level using morphological
112 measurements and observations. The identification of individuals at different life stages was
113 done using the Ege (1939) identification key with amendments by Réveillac *et al.* (2009).
114 The anal-dorsal fin ratio (ratio between anal and dorsal fins in relation to the body length)
115 and back coloration (for adults) or tail pigmentation (for young) were the main morphological
116 characters used to distinguish the four eel species. Non-lethal fin clipping was used to sample
117 individuals for DNA analysis. Larger adult fish were released at their capture site after
118 sampling, but given that smaller fish (< 10 cm total length) are difficult to distinguish
119 morphologically, these individuals were sacrificed and kept as voucher specimens. The fin
120 sample from each individual was stored in labelled vials containing 99% ethanol in a -80°C
121 freezer.

122 DNA was extracted from the individual eel fin clips using the NucleoSpin Tissue kit
123 (Macherey-Nagel), following the manufacturer's standard protocol. A ~600 bp fragment of the
124 COI was amplified using the fish primers FISH-F1 (5'-TCA ACC AAC CAC AAA GAC ATT

125 GGC AC-3') and FISH-R2 (5' -ACT TCA GGG TGA CCG AAG AAT CAG AA 3') from
126 Ward *et al.* (2005). The polymerase chain reactions (PCRs) with a total volume of 12.5 μ L
127 contained: 7 μ L dH₂O, 1 μ L Dream Taq buffer, 0,25 μ L dNTPs, 1 μ L BSA, 0,25 μ L of each
128 primer, 0,05 μ L DreamTaq DNA polymerase (Thermo Fisher Scientific Inc.) and 1 μ L of
129 extracted DNA. The cycling parameters used were: initial denaturation at 95°C (3 min),
130 denaturation at 95°C (30 s), annealing at 52°C (30 s), initial extension at 72°C (1 min) repeated
131 for 34 cycles and, a final extension at 72°C (10 min). PCR products were then Sanger sequenced
132 at the Central Analytical Facility (CAF) at the University of Stellenbosch, Stellenbosch, South
133 Africa, using the same primers.

134 To ensure data quality negative controls were included in all PCRs and all sequences
135 obtained were BLASTed against NCBI GenBank. Similarity scores over 95% were accepted as
136 confirming species identification. All newly generated sequences were deposited in BOLD (See
137 supplementary Table S1 for BOLD accession numbers). Sequences generated in the present
138 study were combined sequences from GenBank (*A. japonica*: HQ339972, MH050933.1,
139 KT355033.1, AB038556.2; *A. bengalensis*: KM875500.1, KF182302.1, MK545096.1; *A.*
140 *bicolor*: KY618771.1, KY618794.1, KY618784.1; *A. marmorata*: MN067970.1, MN067968.1,
141 MN067967.1; *A. mossambica*: AP007244.1) and then aligned with ClustalW 2.1 (Larkin *et al.*
142 2007) and the alignment was optimized manually with Mesquite 3.6 (Maddison and Maddison
143 2018). Since COI is a coding gene no alignment gaps were allowed in the final alignment.
144 *Anguilla japonica* was included as outgroup to root the phylogenies.

145 We conducted phylogenetic analyses using both maximum likelihood (ML) and
146 Bayesian inference (BI). The optimal substitution model for the COI alignment was estimated
147 using the Akaike information criterion (AIC) in jModelTest 2.1.7 (Dariba *et al.* 2012). Garli
148 0.951 (Zwickl 2006) was used to perform maximum likelihood analyses. Branch support was
149 assessed using 1000 bootstrap replicates, with consensus topologies drawn using Phylip 3.695

150 (Felsenstein 2009). Bayesian phylogenies were estimated using MrBayes v3.2 (Ronquist *et al.*
151 2012). Two Bayesian runs, each consisting of four chains, were run for 20 million generations
152 each, with a sampling frequency of 1000. In order to check for convergence of MCMC chains,
153 we used Tracer v1.7.1 (Rambaut *et al.* 2008). The Effective Sample Size (ESS) values of all
154 parameters sampled was >200. The first 20% of trees were removed as burnin before a 50%
155 majority rule consensus tree was drawn. We generated the consensus tree using Phylip 3.695.
156 Trees based on ML and BI were rooted using *A. japonica*.

157 We performed DNA barcode gap analyses by first estimating pair-wise distances among
158 all individuals using the K2P nucleotide substitution model in MEGA 7 (Kumar *et al.* 2016).
159 To determine if the inter- and intra-specific genetic distance classes were separable, we used
160 the Jeffries-Matusita distance (J-M) statistic. We considered the two genetic distance classes
161 statistically separable if $J-M > 1.414$ (Trigg and Flasse 2001).

162 .

163 **RESULTS**

164 A total of 76 fin clips from the four eel species (*A. mossambica*, n = 51; *A.*
165 *marmorata*, n = 17; *A. bengalensis*, n = 6; *A. bicolor*, n = 2) were obtained through
166 opportunistic surveys in the main rivers, tributaries and impoundments in KwaZulu-Natal
167 from 2016 to 2018. Sampling locality details are provided in supplementary information
168 (Table S1).

169 The final aligned, trimmed COI data matrix was 577 bp in length and contained no
170 indels or stop codons. The two optimality criteria (ML and BI) recovered the same topologies
171 and the most likely phylogeny with bootstrap and posterior probability values annotated to
172 branches is shown in Figure 1. The four eel species were recovered as monophyletic. The
173 monophyly of *A. bicolor* and *A. mossambica* was supported with high bootstrap and posterior

174 probability values. The *A. bengalensis* and *A. marmorata* lineages were significantly
175 supported by posterior probabilities but only moderately supported by the bootstrap analysis.

176 The intra-specific (within species) genetic distance ranged from 0 to 0.01, while the
177 inter-specific (between species) genetic distances were much higher and ranged from 0.03 to
178 0.1. The frequency distribution graph (Figure 2) shows that the DNA barcode gap fell
179 between 0.01 to 0.03, with no overlap between inter- and intra-specific genetic distances. A
180 Jeffries-Matusita distance statistic value of 1.997 ($JM > 1.414$) was recovered, confirming
181 that the COI sequences from the four eel species were statistically separable using the COI
182 barcoding technique.

183

184 **DISCUSSION**

185 With molecular techniques becoming more accessible, barcoding is becoming a popular
186 tool for identifying fish species and products (Smith et al., 2008; Cutarelli *et al.* 2014; Helyar
187 *et al.* 2014). The use of this technique for identifying eels has practical applications, especially
188 when it comes to conservation and monitoring of the wildlife trade. Freshwater eel species are
189 a taxon that is particularly vulnerable to changes in the environment, with populations now
190 under threat and highly traded (Jacoby *et al.* 2015). In this regard, barcoding can offer a useful
191 tool for rapid species identification and has been successfully used, for instance, to identify the
192 illegal trade of *A. anguilla* glass eels from Europe into Hong Kong (Stein *et al.* 2016) and also
193 to identify smoked eels species in New Zealand (Smith *et al.* 2008). In southern Africa, the
194 economic interest in eels is currently low, although freshwater eels probably have one of the
195 highest monetary value per weight of any fish product (Jackson, 1976) with smoked European
196 eels fetching 48 euro/kg in 2019 (FAO GLOBEFISH, 2019). Following the ban on freshwater
197 eel exports from the European Union (EU) in 2009, some African countries have entered the
198 global trade, and the endemic *A. mossambica* is now under scrutiny from international investors

199 (Hanzen *et al.* 2019). Here again DNA barcoding could provide useful information on what
200 species of African eel are harvested and traded.

201 Barcode data can be used to improve biodiversity surveys and in so doing significantly
202 contribute towards filling the gap in knowledge on eel reproduction and distribution. For
203 instance, in Malaysia, some specimens morphologically identified as *A. marmorata* were
204 actually confirmed to be *A. bengalensis* through molecular techniques using COI (Arai and
205 Wong, 2016). The latter species *A. bengalensis* had previously not been recorded from this
206 region and would have remained unrecorded without the DNA data. As an extension of classic
207 barcoding using Sanger sequencing, metabarcoding (sequencing of barcodes from bulk samples
208 such as zooplankton) and eDNA (sequencing of barcodes from DNA in environmental samples
209 such as secreted faeces, mucous, gametes, shed skin, hair and carcasses in water) using next-
210 generation sequencing technologies could revolutionize the field of biodiversity research.
211 These techniques could be particularly relevant to species such as eels that have such a long
212 and complex catadromous life cycle, where the tracking of individuals through the many life
213 stages is almost impossible. The utility of metabarcoding in eels has previously been
214 highlighted by Takeuchi *et al.* (2018) which developed a workflow using a 167 bp portion of
215 the ATP6 gene. This latter study did not make use of the standardized COI-barcode, routinely
216 used by the barcoding community, thus reducing broad-scale application. The conformation of
217 the COI-barcode as an appropriate marker for species delimitation for four species of WIO eels,
218 is thus, an important improvement on previous methods and also facilitates the use of public
219 data repositories such as BOLD.

220 While our knowledge of the distribution, ecology and biology of African freshwater eel
221 species remains poor, the present study has shown that barcoding is a method that is rapid and
222 cost-effective and should be considered for further studies in southern Africa. Its suitability also

223 resides in the fact that it is a non-destructive method, applicable to all different life stages, which
224 particularly relevant to species that are vulnerable.

225
226 **CONFLICT OF INTEREST**

227 The authors declare that they have no conflicts of interest.

228
229

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235

236 **REFERENCES**

- 237 Aoyama, J. (2009). Life history and evolution of migration in catadromous eels (Genus
238 *Anguilla*). *Aqua-BioScience Monographs* **2**, 1-42.
- 239 Arai, T., Wong, L.L. (2016). Validation of the occurrence of the tropical eels, *Anguilla*
240 *bengalensis bengalensis* and *A. bicolor bicolor* at Langkawi Island in Peninsular
241 Malaysia, Malaysia. *Tropical Ecology* **57**, 23-31.
- 242 Balon, E.K., (1975). The eels of Lake Kariba: distribution, taxonomic status, age, growth and
243 density. *Journal of Fish Biology* **7**, 797-815.
- 244 Castonguay, M., Durif, C.M. (2015). Understanding the decline in anguillid eels. *ICES Journal*
245 *of Marine Science* **73**, 1-4.
- 246 Cutarelli, A., Amoroso, M.G., De Roma, A., Girardi, S., Galiero, G., Guarino, A., Corrado, F.
247 (2014). Italian market fish species identification and commercial frauds revealing by
248 DNA sequencing. *Food Control* **37**, 46–50.

249 Darriba, D., Taboada, G.L., Doallo, R., Posada, D. (2012). jModelTest 2: more models, new
250 heuristics and parallel computing. *Nature Methods* **9**, 772.

251 Ege, V. (1939). A revision of the genus *Anguilla* Shaw, a systematic, phylogenetic and
252 geographical study. *Dana Report* **16**, 1-256.

253 FAO, 2019. GLOBEFISH European fish price report. Issue 12/2019 December 2019. 20 pp.

254 Felsenstein, J. (2009). PHYLIP (Version 3.69): Phylogeny inference package. *Epub* 3.69.

255 Gagnaire, P.A., Tsukamoto, K., Aoyama, J., Minegishi, Y., Valade, P., Berrebi, P. (2007).
256 RFLP and semi-multiplex PCR-based identification of four eel species from the south-
257 western Indian Ocean region. *Journal of Fish Biology* **71**, 279-287.

258 Hanzen C., Weyl O., Lucas M., Brink K., Downs C., O'Brien G. (2019). Distribution, ecology
259 and status of anguillid eels in East Africa and the Western Indian Ocean, in EELS -
260 biology, monitoring, management, culture and exploitation (Eds P. Coulson and A. Don)
261 pp. 33-58. Proceedings of the International Eels Sciences Symposium, 2017 (5M
262 Publishing, Sheffield.)

263 Hebert, P.D., Cywinska, A., Ball, S.L., Dewaard, J.R. (2003). Biological identifications
264 through DNA barcodes. *Proceedings of the Royal Society of London. Series B* **270**, 313-
265 321.

266 Helyar, S.J., Lloyd, H. ap D., Bruyn, M. de, Leake, J., Bennett, N., Carvalho, G.R. (2014).
267 Fish Product Mislabelling: Failings of Traceability in the Production Chain and
268 Implications for Illegal, Unreported and Unregulated (IUU) Fishing. *PLoS One* **9**,
269 e98691.

270 Jackson, P.B.N. (1976). The distribution and growth of eels of the genus *Anguilla* Shaw, 1830,
271 in the Western Indian Ocean. *Travaux et Documents de l'Office de la Recherche*
272 *Scientifique et Technique d'Outre-Mer*, **47**, 189-192

273 Jacoby, D.M.P., Casselman, J.M., Crook, V., DeLucia, M.-B., Ahn, H., Kaifu, K., Kurwie, T.,
274 Sasal, P., Silfvergrip, A.M.C., Smith, K.G., Uchida, K., Walker, A.M., Gollock, M.J.
275 (2015). Synergistic patterns of threat and the challenges facing global anguillid eel
276 conservation. *Global Ecology and Conservation* **4**, 321-333.

277 Jespersen, P. (1942). Indo-Pacific leptocephalids of the genus *Anguilla*: systematic and
278 biological studies. *Dana Report Carlsberg Foundation*, **22**, 1-128

279 Jubb, R.A. (1961). An illustrated guide to the freshwater fishes of the Zambezi River, Lake
280 Kariba, Pungwe, Sabi, Lundi and Limpopo Rivers. Stuart Manning, Cape Town.

281 Kaifu, K., Stein, F., Dekker, W., Walker, N., Dolloff, A., Steele, K., Aguirre A.A., Nijman, V.,
282 Siritwat, P., Sasal, P. (2019). Global exploitation of freshwater eels (genus *Anguilla*):
283 fisheries, stock status and illegal trade, in EELS - biology, monitoring, management,
284 culture and exploitation (Eds P. Coulson and A. Don) pp. 377-422. Proceedings of the
285 International Eels Sciences Symposium, 2017 (5M Publishing, Sheffield.)

286 Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7 (Version 7.0): Molecular Evolutionary
287 Genetics Analysis. Available at <https://www.megasoftware.net>

288 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.,
289 Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins,
290 D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948.

291 Lecomte-Finiger, R. (2003). The genus *Anguilla* Schrank, 1798: Current state of knowledge
292 and questions. *Reviews in Fish Biology and Fisheries* **13**, 265-279.

293 Maddison, W. P., Maddison, D.R. (2018). Mesquite (Version 3.51): a modular system for
294 evolutionary analysis. Available at <http://www.mesquiteproject.org>.

295 Neiffer, D.L., Stamper, M.A. (2009). Fish Sedation, Anesthesia, Analgesia, and Euthanasia:
296 Considerations, Methods, and Types of Drugs. *ILAR Journal* **50**, 343-360.

297 Pous, S., Feunteun, E., Ellien, C. (2010). Investigation of tropical eel spawning area in the
298 South-Western Indian Ocean: Influence of the oceanic circulation. *Progress in*
299 *Oceanography* **86**, 396-413.

300 Radulovici, A.E., Archambault, P., Dufresne, F., 2010. DNA Barcodes for Marine
301 Biodiversity: Moving Fast Forward? *Diversity* **2**, 450-472.

302 Rahman, S., Barua, E., Choudhury, J.K., Duta, A., Kalita, M.C. (2015). Generation of DNA
303 barcodes in Indian mottled eel (*Anguilla bengalensis*): A threatened ichthyofauna of
304 Assam, India. *International Journal of Agriculture, Environment and Biotechnology* **8**,
305 511-519.

306 Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A. (2018). Posterior
307 summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**, 901-
308 904.

309 Réveillac, É., Gagnaire, P.-A., Lecomte-Finiger, R., Berrebi, P., Robinet, T., Valade, P.,
310 Feunteun, É. (2009). Development of a key using morphological characters to distinguish
311 south-western Indian Ocean anguillid glass eels. *Journal of Fish Biology* **74**, 2171-2177.

312 Robinet, T., Feunteun, E., Keith, P., Marquet, G., Olivier, J.-M., Réveillac, E., Valade, P.
313 (2007). Eel community structure, fluvial recruitment of *Anguilla marmorata* and
314 indication for a weak local production of spawners from rivers of Réunion and Mauritius
315 islands. *Environmental Biology of Fishes* **78**, 93-105.

316 Robinet, T., Réveillac, E., Kuroki, M., Aoyama, J., Tsukamoto, K., Rabenevanana, M.W.,
317 Valade, P., Gagnaire, P.-A., Berrebi, P., Feunteun, E. (2008). New clues for freshwater
318 eels (*Anguilla* spp.) migration routes to eastern Madagascar and surrounding islands.
319 *Marine Biology* **154**, 453-463.

320 Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B.,
321 Liu, L., Suchard, M.A. and Huelsenbeck, J.P. (2012). MrBayes 3.2: Efficient Bayesian

322 phylogenetic inference and model choice across a large model space. *Systematic Biology*
323 **61**, 539-42.

324 Skelton, P.H. (2001). A complete guide to the freshwater fishes of southern Africa. Struik,
325 Cape Town.

326 Smith, P.J., McVeagh, S.M., Steinke, D. (2008). DNA barcoding for the identification of
327 smoked fish products. *Journal of Fish Biology* **72**, 464-471.

328 Stein, F.M., Wong, J.C., Sheng, V., Law, C.S., Schröder, B., Baker, D.M. (2016). First genetic
329 evidence of illegal trade in endangered European eel (*Anguilla anguilla*) from Europe to
330 Asia. *Conservation Genetics Resources* **8**, 533-537.

331 Takeuchi, A., Sado, T., Gotoh, R.O., Watanabe, S., Tsukamoto, K., Miya, M. (2019). New
332 PCR primers for metabarcoding environmental DNA from freshwater eels, genus
333 *Anguilla*. *Scientific Reports* **9**, 1-11.

334 Trigg, S., Flasse, S. (2001). An evaluation of different bi-spectral spaces for discriminating
335 burned shrub-savannah. *International Journal of Remote Sensing* **22**, 2641-2647.

336 Valade, P., Hoarau, P., Bonnefoy, A. (2018). Plan Directeur de Conservation en faveur des
337 Anguillidae à l'île de La Réunion 2018-2027. DEAL Réunion (Direction de
338 l'environnement, de l'aménagement et du logement: [www.reunion.developpement-](http://www.reunion.developpement-durable.gouv.fr)
339 [durable.gouv.fr](http://www.reunion.developpement-durable.gouv.fr)).

340 Ward, R., Zemplak, T., Innes, B., Last, P., Hebert, P. (2005). DNA barcoding Australia's fish
341 species. *Philosophical Transactions of the Royal Society B: Biological Sciences*.
342 **360**, 1847-1857.

343 Wasserman, R.J., Weyl, O.L.F., Strydom, N.A. (2011). The effects of instream barriers on the
344 distribution of migratory marine-spawned fishes in the lower reaches of the Sundays
345 River, South Africa. *Water SA* **37**, 495-504.

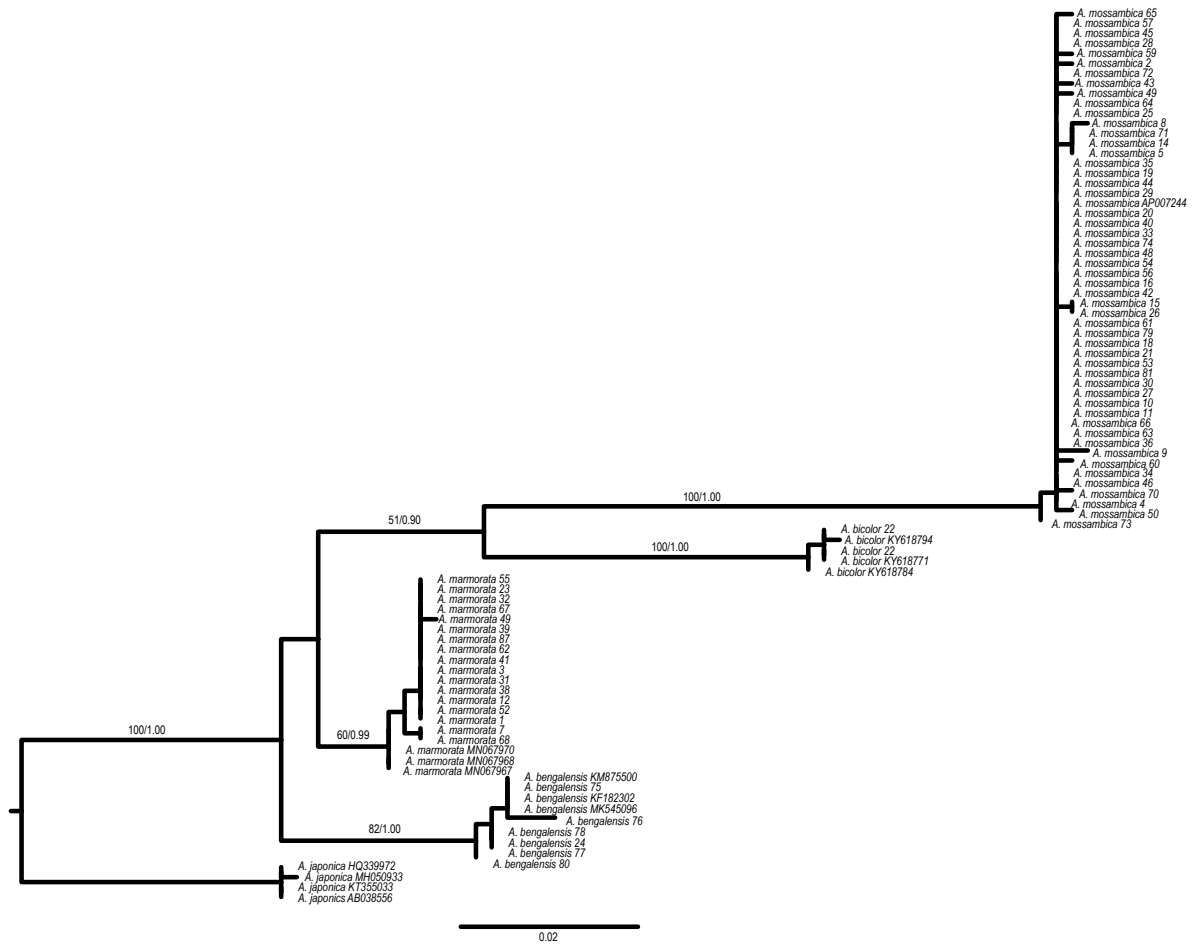
346 Watanabe, S., Aoyama, J., Tsukamoto, K. (2004). Reexamination of Ege's (1939) use of
347 taxonomic characters of the genus *Anguilla*. *Bulletin of Marine Science* **74**, 337-351.

348 Zwickl, D.J. (2006). Genetic algorithm approaches for the phylogenetic analysis of large
349 biological sequence datasets under the maximum likelihood criterion. PhD thesis,
350 University of Texas, Austin.

351

352

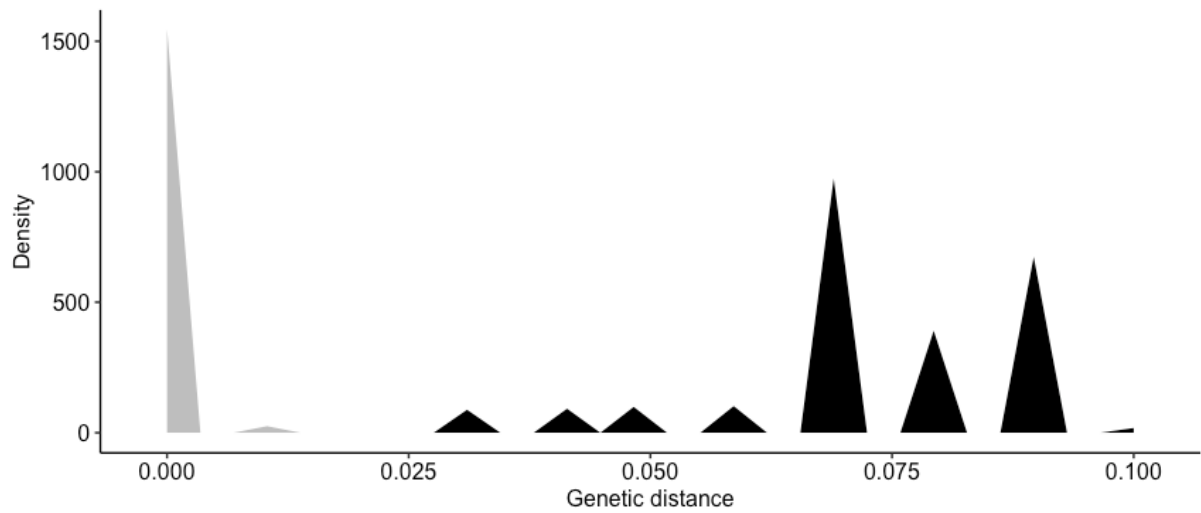
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355

356 **Figure 1.** Maximum likelihood phylogeny inferred from CO1 sequences for the four African
 357 eel species (*Anguilla mossambica*, *A. bicolor*, *A. bengalensis*, and *A. marmorata*). The
 358 Japanese eel *A. japonica* was used as an outgroup. Maximum likelihood bootstrap / Bayesian
 359 posterior probability values are shown on branches. Only bootstrap values > 60% and posterior
 360 probability values > 0.70 are shown. Specimen numbers correspond to that provided in Table
 361 S1. Accession numbers are provided for sequences taken from GenBank

362



363

364 **Figure 2.** Frequency distribution of intra- and inter-specific K2P pairwise distances
 365 calculated from CO1 sequences for the four African eel species (*Anguilla mossambica*, *A.*
 366 *bicolor*, *A. bengalensis*, and *A. marmorata*) in the present study. Intra-specific distances are
 367 shown in light grey while inter-specific distances are shown in black. The DNA barcode gap
 368 lay between 0.01 and 0.03.

369

370 **Supplementary information**

371 **Supplementary information Table S1.** Summary of collection information for eel

372 specimens included in the present study. Specimens were collected from main river systems
 373 in KwaZulu-Natal province (South Africa) from 2016 - 2018. Data presented include year of
 374 collection, geographical location of specimen (latitude and longitude), river and catchment
 375 where sample was obtained as well as the BOLD accession number.

Individual	Year collected	Latitude	Longitude	River	Catchment	BOLD accession
A. mossambica 72	2017	-30.594629	29.793018	Weza	Mtamvuna	FBIPA039-18
A. mossambica 79	2017	-29.84311	30.5516	Tala Dam	Mlazi	FBIPA054-18
A. mossambica 14	2017	-29.769772	30.829056	Molweni Ronald Kloof stream	Umgeni	FBIPA033-18
A. mossambica 83	2017	-29.779781	30.834492		Umgeni	FBIPA077-18
A. mossambica 71	2017	-29.50717	31.2285643	Golf dam	Simbithi	FBIPA038-18
A. mossambica 27	2017	-29.773137	30.798678	Molweni	Umgeni	FBIPA032-18
A. mossambica 65	2017	-28.118332	32.185545	Hluhluwe	Hluhluwe	FBIPA010-18
A. mossambica 74	2017	-30.579953	29.787291	Mzimkhulwana	Mzimkhulu	FBIPA056-18
A. mossambica 4	2017	-29.809722	30.5	Mlazi	Mlazi	FBIPA004-18
A. mossambica 70	2017	-29.50717	31.2285643	Golf dam	Simbithi	FBIPA057-18
A. marmorata 62	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA041-18
A. mossambica 34	2017	-29.1711	31.42149	Mandini	Thukela	FBIPA021-18
A. bengalensis 76	2017	-28.703679	30.0487	Thukela	Thukela	FBIPA055-18
A. mossambica 10	2016	-28.94142	31.39416	Nwaku	Matikulu	FBIPA009-18
A. mossambica 66	2017	-29.422257	31.25084	Palm lake dam	Mhlalali	FBIPA037-18
A. mossambica 28	2016	-29.911667	30.2219444	Mkobeni trib.	Mkomazi	FBIPA006-18
A. mossambica 40	2016	-28.94142	31.39416	Nwaku	Matikulu	FBIPA014-18
A. marmorata 55	2016	-28.925444	31.6424222	uMlalazi	uMlalazi	FBIPA053-18
A. marmorata 3	2017	-29.17056	31.421092	Mandini	Thukela	FBIPA049-18
A. mossambica 30	2017	-29.89894	30.06294	Umkomaas	Mkomazi	FBIPA070-18
A. mossambica 25	2016	-30.423944	29.9155556	Bisi	Umzimkhulu	FBIPA005-18
A. marmorata 23	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA029-18
A. mossambica 9	2016	-28.94142	31.39416	Nwaku	Matikulu	FBIPA017-18
A. marmorata 41	2017	-29.17056	31.421092	Mandini	Thukela	FBIPA023-18
A. mossambica 2	2017	-29.868727	30.780983	Mlazi	Mlazi	FBIPA001-18
A. bengalensis 24	2016	-28.3596	31.99434	Imfolozi	Imfolozi	FBIPA008-18
A. marmorata 39	2016	-29.17056	31.421092	Mandini Thukela	Thukela	FBIPA030-18
A. marmorata 52	2017	-29.169146	31.375128	(fishway)	Thukela	FBIPA062-18
A. mossambica 44	2017	-29.12901	31.32412	Nembe	Thukela	FBIPA024-18
A. mossambica 20	2017	-29.08235	31.35244	Nembe	Thukela	FBIPA020-18
A. mossambica 36	2017	-28.904211	30.418753	Mooi	Thukela	FBIPA019-18
A. mossambica 33	2017	-29.89894	30.06294	Umkomaas	Mkomazi	FBIPA068-18

A. mossambica 15	2016	-30.423944	29.9155556	Bisi	Umzimkhulu	FBIPA002-18
A. mossambica 57	2017	-29.364997	31.290369	Nchaweni	Mvoti	FBIPA022-18
A. mossambica 21	2016	-28.94142	31.39416	Nwaku	Matikulu	FBIPA012-18
A. mossambica 73	2017	-28.719424	30.06529	Thukela	Thukela	FBIPA025-18
A. mossambica 16	2016	-28.94142	31.39416	Nwaku	Matikulu	FBIPA016-18
A. mossambica 29	2016	-30.220538	30.504744		Mpambanyoni	FBIPA013-18
A. mossambica 11	2016	-28.756331	30.150376	Thukela	Thukela	FBIPA074-18
A. mossambica 18	2016	-27.803441	30.247932	Buffalo	Thukela	FBIPA073-18
A. mossambica 26	2016	-30.423944	29.9155556	Bisi	Umzimkhulu	FBIPA071-18
A. mossambica 45	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA043-18
A. mossambica 48	2016	-29.170824	31.393199	Thukela	Thukela	FBIPA065-18
A. mossambica 61	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA046-18
A. mossambica 43	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA048-18
A. mossambica 59	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA044-18
A. marmorata 31	2016	-28.925444	31.6424222	uMlalazi	uMlalazi	FBIPA003-18
A. mossambica 42	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA042-18
A. mossambica 19	2017	-29.161057	31.336045	Thukela	Thukela	FBIPA072-18
A. marmorata 12	2016	-28.74695	31.74745	Mhlatuze	Matikulu	FBIPA011-18
A. mossambica 35	2017	-29.167362	31.335648	Thukela	Thukela	FBIPA067-18
A. mossambica 46	2017	-29.167362	31.335648	Thukela	Thukela	FBIPA078-19
A. mossambica 50	2017	-29.167362	31.335648	Thukela	Thukela	FBIPA064-18
A. mossambica 53	2017	-29.167362	31.335648	Thukela	Thukela	FBIPA050-18
A. marmorata 7	2016	-28.925444	31.6424222	uMlalazi	uMlalazi	FBIPA075-18
A. mossambica 60	2017	-29.167362	31.335648	Thukela	Thukela	FBIPA060-18
A. marmorata 1	2017	-28.119305	32.183157	Hluhluwe	Hluhluwe	FBIPA034-18
A. mossambica 63	2016	-29.559913	31.174085	Tongati	Tongati	FBIPA059-18
A. mossambica 64	2016	-29.559913	31.174085	Tongati	Tongati	FBIPA058-18
A. marmorata 69	2017	-29.515525	31.2146688	Heron dam	Simbithi	FBIPA026-18
A. marmorata 87	2017	-28.23146	31.1883	Imfolozi	Imfolozi	FBIPA007-18
A. mossambica 49	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA047-18
A. mossambica 54	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA045-18
A. marmorata 68	2017	-29.515525	31.2146688	Heron dam	Simbithi	FBIPA036-18
A. marmorata 67	2017	-29.516295	31.2119255	Weaver dam	Simbithi	FBIPA035-18
A. bengalensis 78	2017	-28.715484	30.06213	Thukela	Thukela	FBIPA027-18
A. mossambica 47	2016	-29.17056	31.421092	Mandini	Thukela	FBIPA052-18
A. bengalensis 77	2017	-28.710651	30.060804	Thukela	Thukela	FBIPA040-18
A. marmorata 38	2017	-29.169475	31.395291	Thukela	Thukela	FBIPA031-18
A. bengalensis 75	2017	-28.702976	30.054005	Thukela	Thukela	FBIPA028-18
A. bengalensis 80	2017	-28.715484	30.06213	Thukela	Thukela	FBIPA051-18
A. marmorata 32	2017	-28.119305	32.183157	Hluhluwe	Hluhluwe	FBIPA069-18
A. mossambica 5	2017	-29.82478	30.924995	Palmiet	Umgeni	FBIPA076-18
A. mossambica 37	2016	-30.220538	30.504744		Mpambanyoni	FBIPA066-18
A. mossambica 56	2016	-28.756331	30.150376	Thukela	Thukela	FBIPA061-18
A. mossambica 81	2017	-29.720833	30.903937	Umgeni	Umgeni	FBIPA018-18

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