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

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Running header: African freshwater eel DNA barcoding

ABSTRACT

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

KEYWORDS

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

INTRODUCTION

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

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

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

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

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

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

METHODS

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

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

GGC AC-3′) and FISH-R2 (5′ -ACT TCA GGG TGA CCG AAG AAT CAG AA 3′) from Ward *et al.* (2005). The polymerase chain reactions (PCRs) with a total volume of 12.5 µL contained: 7 µL dH20, 1 µL Dream Taq buffer, 0,25 µL dNTPs, 1 µL BSA, 0,25 µL of each primer, 0,05 µL DreamTaq DNA polymerase (Thermo Fisher Scientific Inc.) and 1 µL of extracted DNA. The cycling parameters used were: initial denaturation at 95°C (3 min), denaturation at 95°C (30 s), annealing at 52°C (30 s), initial extension at 72°C (1 min) repeated for 34 cycles and, a final extension at 72°C (10 min). PCR products were then Sanger sequenced at the Central Analytical Facility (CAF) at the University of Stellenbosch, Stellenbosch, South Africa, using the same primers.

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

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

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

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

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RESULTS

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

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

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

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

DISCUSSION

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

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

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

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

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

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Figures

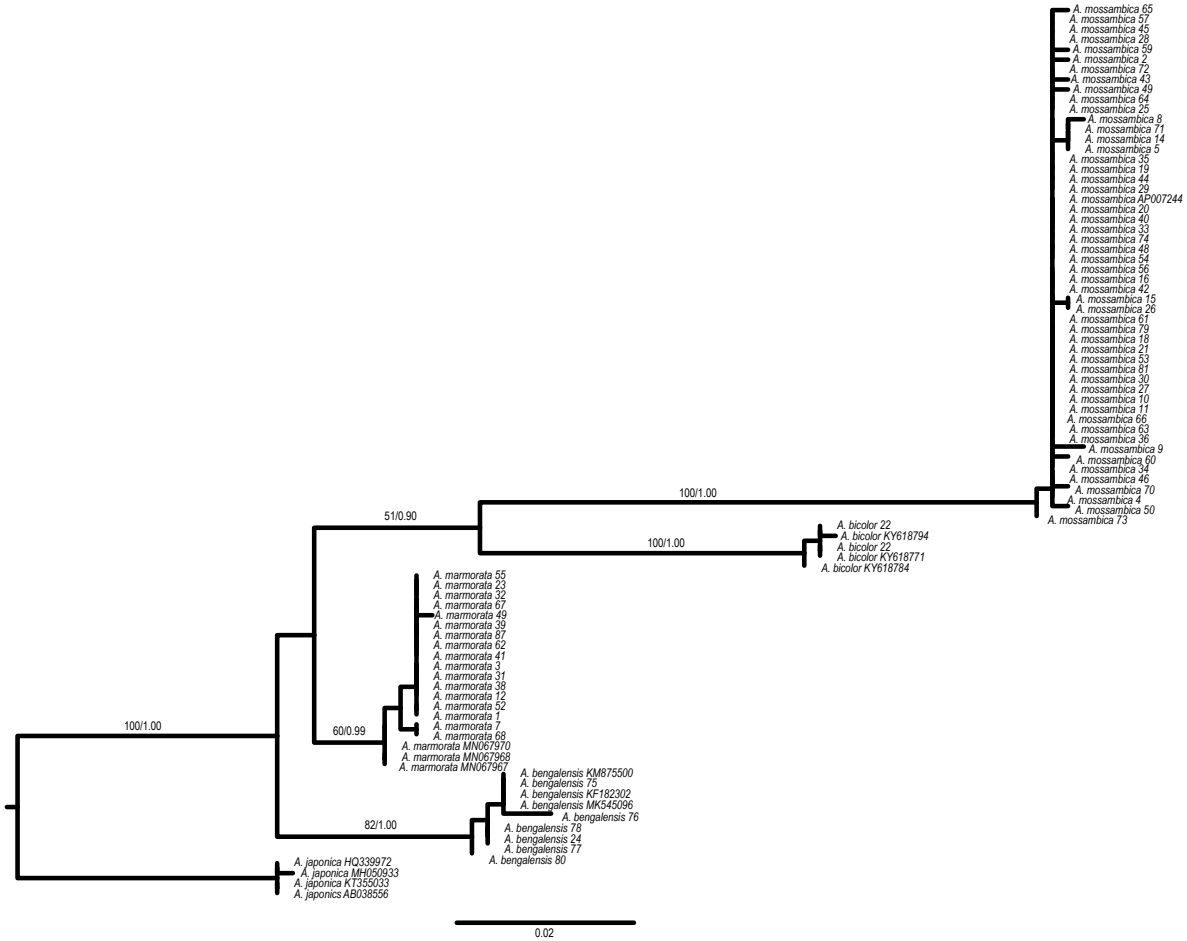

Figure 1. Maximum likelihood phylogeny inferred from CO1 sequences for the four African eel species (*Anguilla mossambica*, *A. bicolor*, *A. bengalensis*, and *A. marmorata*). The Japanese eel *A. japonica* was used as an outgroup. Maximum likelihood bootstrap / Bayesian posterior probability values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

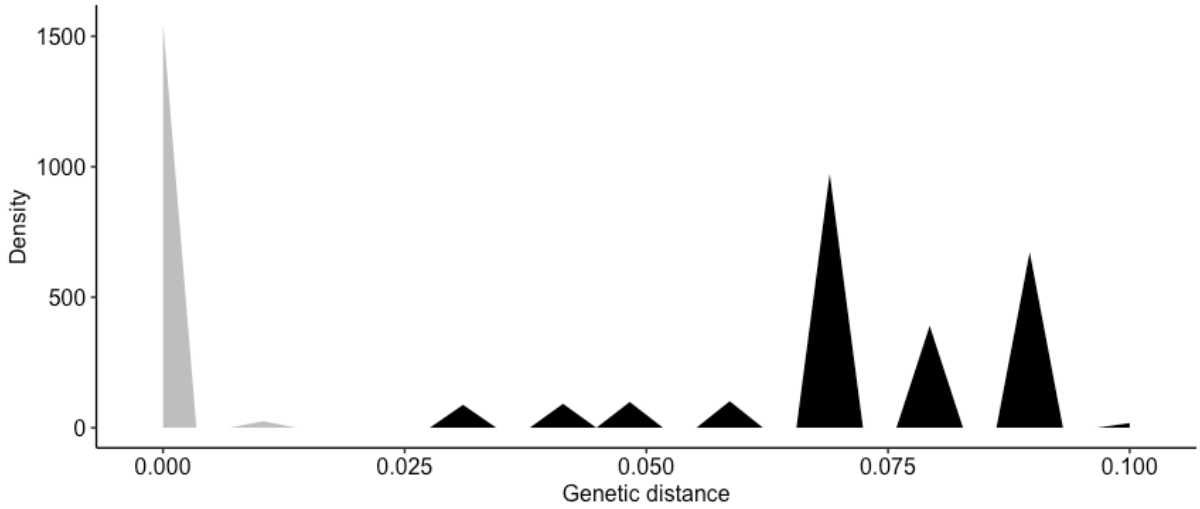

Figure 2. Frequency distribution of intra- and inter-specific K2P pairwise distances calculated from CO1 sequences for the four African eel species (*Anguilla mossambica*, *A. bicolor*, *A. bengalensis*, and *A. marmorata*) in the present study. Intra-specific distances are shown in light grey while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

Supplementary information

Supplementary information Table S1. Summary of collection information for eel specimens included in the present study. Specimens were collected from main river systems in KwaZulu-Natal province (South Africa) from 2016 - 2018. Data presented include year of collection, geographical location of specimen (latitude and longitude), river and catchment 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	Umgeni	FBIPA033-18
A. mossambica 83	2017	-29.779781	30.834492	Ronald Kloof stream	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	FBIPA030-18
A. marmorata 52	2017	-29.169146	31.375128	Thukela (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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