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A genomic toolbox for population assignment and monitoring of a conservation dependent Amazonian crocodylian (*Melanosuchus niger*: Alligatoridae: Crocodylia)

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Abstract: We developed a set of 78 SNP markers using ddRADseq, to assign individuals of the black caiman to one of four geographic areas in the Central Amazon. With a simple genotyping protocol, it is possible to assign individuals an area of origin at $p < 0.05$. The protocol thus provides a useful tool for monitoring the geographic origin of individuals and commercialized subproducts.

Keywords: ddRADs, SNPs, conservation genomics, Amazon, black caiman

Resumen. Una herramienta genómica para la asignación poblacional y monitoreo de un cocodriliano amazónico dependiente de conservación (*Melanosuchus niger*: Alligatoridae: Crocodylia). Desarrollamos un set de 78 marcadores SNP usando ddRADseq, para asignar individuos del caimán negro a una de cuatro áreas geográficas en la Amazonia Central. A través de un protocolo simple de genotipaje, es posible asignar individuos a su área de origen con $p < 0.05$. Por lo tanto, este protocolo proporciona una herramienta útil para monitorear la procedencia geográfica de individuos y subproductos comercializados.

Palabras clave: ddRADs, SNPs, genómica de la conservación, Amazonia, caimán negro

Understanding the factors that lead to the current conservation status of an endangered species is the most important goal of conservation genetics (DeSalle & Amato 2004). Hunting and trade are the principal causes that affect populations of most large species, resulting in over-exploitation driven declines. Molecular approaches have been shown to be very useful in estimating a variety of parameters that reveal the prejudicial effects of over-exploitation of populations and in contributing to taking management and conservation decisions. To enforce conservation legislation, it is essential to identify distinct populations or taxonomic units; thus an effective and efficient method is required to detect, monitor and control the trade in wildlife (Yan *et al*. 2005, Eaton *et al*. 2010). Molecular tools are one of the most important and useful method than provides a solution, allowing the use of DNA as a "fingerprint". Using these tools, it is possible to obtain population profiles to assign or match individuals to areas of their origin (DeSalle & Amato 2004, Allendorf *et al*. 2010). Traditionally, mitochondrial markers and microsatellites have been used to this porpoise, but new sequencing technologies and protocols have enabled rapid and efficient development of genomic markers, such as

SNPs (Single Nucleotide Polymorphisms), to address important conservation issues at a fine population level in non-model species (Allendorf *et al*. 2010). Crocodilians have been severely affected by indiscriminate and commercial hunting. One of the species that has a long history of exploitation is *Melanosuchus niger*, an Amazonian big predator. The commercial hunting for its leather began in the 1930s, that led to population declines and local extinctions, and continued through the 1980s despite prohibitions of commercial hunting (Plotkin *et al*. 1983, Rebêlo & Magnusson 1983, Da Silveira & Thorbjarnarson 1999). Beginning in the 1990s, illegal hunting shifted to supply the illicit trade with dried/salted meat and at the end of this decade, its meat also started to be used as a bait in the piracatinga (*Calophysus macropterus*) fishery (Da Silveira 2003, Marioni *et al*. 2013); both activities continue to this day. Nevertheless, the black caiman has increased its numbers in the last years in a number of areas within its original distribution (Da Silveira & Thorbjarnarson 1999, Thorbjarnarson 2010, Marioni *et al*. 2013), and for that reason, the species was reclassified in 2000 to the LR/CD (Low Risk/Conservation Dependent) category in the IUCN Red List of Threatened Species (Ross 2000). It is included on CITES Appendix I, except in Brazil and Ecuador where is listed in Appendix II (Thorbjarnarson 2010). Only few molecular studies of *M. niger* have been published to date (Farias *et al*. 2004, De Thoisy *et al*. 2006, Vasconcelos *et al*. 2008, Muniz *et al*. 2011), but no genomic data are available for the species. Many genomic studies have been recently published with conservation approaches for other species: Houston *et al*. (2015) validated SNP assays to asses population demographic parameters of the burying beetle (*Nicrophorus orbicollis*); Martínez *et al*. (2016) developed SNPs to identify gilded catfish (*Brachyplatystoma rousseauxii*) from Orinoco and Amazonas basins; Stetz *et al*. (2016) discovered SNPs to assign individuals of North American river otter (*Lontra canadensis*) to population of origin; Kleinman-Ruiz *et al*. (2017) identified a SNP panel to genetic management and non-invasive monitoring of Iberian lynx (*Lynx pardinus*); Carvalho *et al*. (2017) generated SNPs to identify individuals of the side-necked turtle (*Phrynops geoffroanus*) from different lineages. This is thus the first study to develop SNP markers to identify individuals from different populations with the goal of contributing with the control of trade in wildlife, with emphasis on *M. niger*, a commonly affected species by the illegal hunting.

In this study, we used next-generation sequencing (NGS) to develop SNP markers for monitoring *M. niger* from four localities in the central Amazon; two of them are from conservation units integrated within the Central Amazon Conservation Complex: 1) Anavilhanas National Park (Negro River) and 2) Mamirauá SDR (Japura River), and the other two localities are from areas with no formal protection: 3) Janauacá Lake (Amazon River) and 4) the lower part of the Madeira River (Fig. 1); the two non-protected areas have high incidences of illegal hunting and exploitations. Total DNA from 32 samples (Anavilhanas: ten; Mamirauá: ten; Janauacá: six; Madeira: six) was extracted using the 2% CTAB protocol (Doyle & Doyle 1987). To construct the genomic library, we followed the double-digest restriction site-associated DNA (ddRAD) method proposed by Peterson *et al*. (2012), but with modifications as described in a public GitHub repository hosted at https://github.com/legalLab/protocols-scripts, using two restriction enzymes, SdaI (rare 8-cutter) and Csp6I (common 4-cutter), and ligating two adapters, P (Ion Torrent) and A (with barcode). All the samples were amplified and then pooled together equimolarly and size selected for a range 374-456 bp. Finally, the product was sequenced using Ion Torrent NGS Technology (Thermo Fisher Scientific). The raw reads were processed in the pipeline Stacks v1.42 (Catchen *et al*. 2013) using three components. We implemented process_radtags to filter sequences based on quality thresholds and to demultiplex by barcodes. Due to the lack of reference genome, we used denovo_maps.pl to built loci and to call SNPs, with a 4X minimum depth for allele identification, and then to create a catalog with all data. To compute the genetic statistics within and between locations, we ran populations considering the presence of each locus in at least 4 populations and minimum in 50% of individuals per population, and with a minor allele frequency of 0.01.

After processing data in Stacks, 5.434.361 reads were retained. From these, we obtained 43.186 tags corresponding to 782 loci, of which we selected those with population specific (private) alleles: 123 for Anavilhanas, 99 for Mamirauá, 56 for Janauacá and 76 for lower Madeira River. From these, we chose a set that allowed an assignment of an individual to its respective population with a probability of 95%. These sets comprised 17 loci (Anavilhanas), 33 loci (Mamirauá), 22 loci

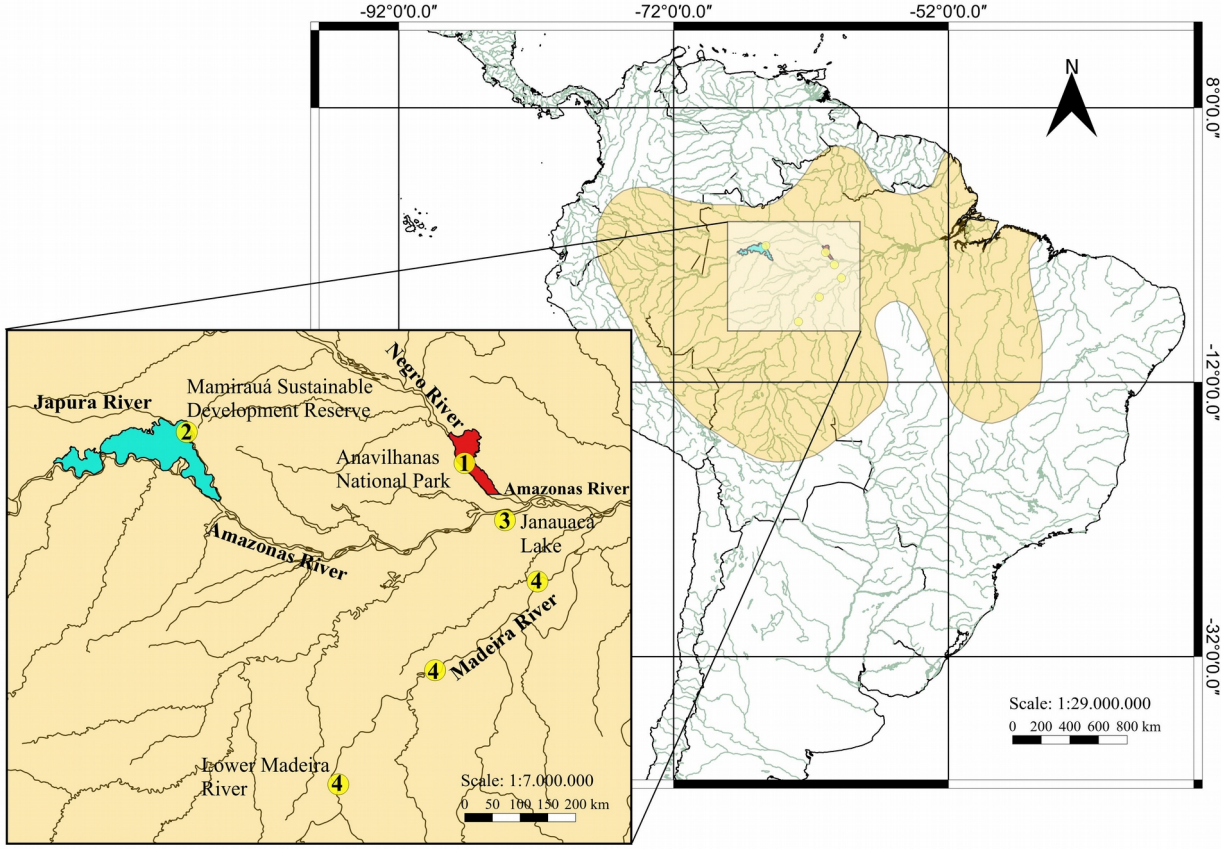

Figure 1. Map of sample localities. Colour area delimits the distribution of the Black Caiman, *Melanosuchus niger*, in Amazon region, South America. The close-up shows the molecular sampling area of central Amazon with study points in yellow circles: 1) Anavilhanas; 2) Mamirauá; 3): Janauaca, and 4) lower Madeira. (Distribution shapefile: IUCN -International Union for Conservation of Nature. 2014. *Melanosuchus niger*. The IUCN Red List of Threatened Species. Version 2016,3. http://www.iucnredlist.org. Downloaded on 02 November 2016).

(Janauacá) and 6 loci (Lower Madeira River) (Table I). Based on the consensus tag sequences (GenBank ID KY568011-KY568088), we designed one of the primers for each locus using the Batchprimer3 tool plugin (You *et al*. 2008) of the Geneious software 10.0.7 (Kearse *et al*. 2012) and the other one manually, with the 3' end of the primer ending on the SNP. Such design allows for a simple genotyping protocol, with presence of an amplicon indicating the presence of a SNP, and the absence of the amplicon an absence of the SNP. Detection of one or more amplicons implied that an individual originates from that particular locality, while rejecting an assignment of an individual to a particular locality due to lack of detection of an amplicon occurs with less than 5% probability (alpha error < 0.05).

The results show that the set of designed primers could identify individuals of the same species from different places or even parts or commercial product from them, economically and quickly, because of the presence of private alleles for each population. This is very important for use in wildlife conservation and management because it allows institutions responsible for management and wildlife regulation enforcement to determine if a sample (skin, meat, whole animal) has an illegal origin or not (Eaton *et al*. 2010), in our case if the sample is from a protected area with a management plan or from a place without formal protection but subject to intensive illegal exploitation. In this sense, it is important because it allows to monitor populations to guarantee that declines do not happen again, and in the case of conservation dependent species that the recovery process and thus the conservation of the species proceeds successfully.

In conclusion, we developed a set of SNP markers that are useful to determine a population profile and to assign individuals to their locality of origin with high accuracy. We also expect these SNPs become an important and widely used tool for implementing sustainable use and management practices of *M. niger*, and for certifying the origin of individuals and derivative products.

Table I. SNP markers for molecular identification and assignment of individual from four geographical localities of *Melanosuchus niger* from the central Amazon.

Locus ID	Locality	Primer (5' – 3')	Position	Alleles	Q frequency	Product size	GenBank accession number
418	Ana	F-GTTCTGTTTTAACTGTTGCCC R-GGGAGAGATGCCAGGAAGAC	68	T/C	0.16666667	138	KY568011
730	Ana	F-ACCACTGTGCTGCCCAATAA R-CCTGGTCCCGAGAGAGAGAAGCA	102	C/T	0.14285714	116	KY568012
2024	Ana	F-CAAGGTGATTACAGACAGGAGCA R-CAGAGATGGCAGGCAAGTCA	27	G/A	0.125	163	KY568013
2504	Ana	F- TTTACATGTGTTCCAGGAGC R-TCAAAGTGCCTTGTCCCCAT	42	G/C	0.25	129	KY568014
3130	Ana	F-TGTTATGGCCAGCATCCTA R-GCAGAGCCACTCAAACACTC	55	G/A	0.14285714	151	KY568015
3443	Ana	F-CTCACAAGATCCACATGAATG R-AATGATTATCTTACGCTGGTT	85	A/G	0.125	116	KY568016
3571	Ana	F-TTAATTACAGCTTTCTCTTTCCT R-CACAAACCAGGGAATGCTGC	30	C/T	0.125	187	KY568017
4290	Ana	F-GAGAGAAGGAGAGAGAGGCAG R-GGTCTTCAGTCTGATTTTCCCCT	90	A/G	0.25	126	KY568018
4693	Ana	F-ACTCCAATCATTTGTTGCCGTC R-TGAGAGCCCATTAGACTGATT	145	C/A	0.125	121	KY568019
5009	Ana	F-CTACATGATATATCAGATATCAT R-AGGTGGGAAAGGGAAAAGTGT	37	C/T	0.14285714	133	KY568020
5818	Ana	F-ATGTTAAATTGTGCAAAGCCG R-ACTGGGTAACTGATAGGTCTCA	67	A/G	0.14285714	109	KY568021
6402	Ana	F-TGCAAATACATGATTTTTAC R-GAGCCCCTGTATCACCAGAC	36	T/C	0.125	145	KY568022
6463	Ana	F-AGAAGCAAAAAGCTGCTGCGA R-CTGTGTGAACATGGCTCGGA	65	T/A	0.125	109	KY568023
6663	Ana	F-ATGCAATGACCCCCTTCCAC R-GAACAGACTCACCACCAGTGGC	121	A/G	0.14285714	116	KY568024
9019	Ana	F-GCCACATGAAGAGAGGTTGAAAG R-AGATGCTCTCCGAGCCCCTA	152	C/T	0.28571429	130	KY568025
10891	Ana	F-GCAATCAGGTTTGTGGGCACTGG R-TTATTTGTGTTATTAGGCAGTG	93	T/G	0.16666667	114	KY568026
11619	Ana	F-CACAATGACCCCTTTCCACTGC R-GCTGTGTAGAAGAGCTTT	110	G/A	0.14285714	100	KY568027
194	Mam	F-CTCCCCAGGTGAAATCTTGGT R-ACAGGATCAAATCTTGTTGATA	143	C/T	0.05555556	119	KY568028
203	Mam	F-AAAGGGGAAGACAGTTACAT R-TGCTGTATGACTATGAAAAGGTCT	97	C/T	0.07142857	108	KY568029
356	Mam	F-CCTCAGGTCGCCTATCACGC R-AGTCAGCCAACTTCTTTGCAGC	30	T/C	0.05555556	128	KY568030
658	Mam	F-TGGTGGCAGAACAGTCCCTA R-CAAATTCTGTCCTGGAGTCT	156	T/A	0.05555556	137	KY568031
923	Mam	F-CGGAGGGCTGACTTTGACAT R-CGAGATGGAATAGCCTTAGCC	133	A/G	0.05555556	142	KY568032
1004	Mam	F-ACTGCTGTGCCTGCCTTAAA R-TTCCAGCTCTAATGTCTATGG	141	T/C	0.05555556	160	KY568033

Locus ID	Locality	Primer (5' – 3')	Position	Alleles	Q frequency	Product size	GenBank accession number
1106	Mam	F-ACAGTGTGACTAGCCTAC R-GCACATGATGGCAGAGGGAG	92	T/C	0.05555556	105	KY568034
1535	Mam	F-TGATTATGCCCTGTCTCAAG R-AGCTGGAGCTGGGACAGGCTGC	146	A/G	0.11111111	151	KY568035
1819	Mam	F-ATTGAATACCAGTGAGTCCCTG R-TCAGAGTTACTTTAATGCTAA	144	G/T	0.08333333	111	KY568036
1862	Mam	F-TAAGATGTATTGGTGGCCCTAG R-CCTGCCCCAACTACCAAAAGAC	154	A/G	0.07142857	149	KY568037
1901	Mam	F-CGGGTCCTTTCCTGGCCTGA R-CCTGGTAGTTGGGGTGGCA	40	G/A	0.07142857	148	KY568038
2125	Mam	F-CGTATGTCTACACGTGGTGGA R-GGCACTTCACTTGATACCGAA	156	G/T	0.07142857	160	KY568039
2506	Mam	F-GCCATGTTACTCTTGAATTC R-ATTGAATGCTCAGCAAGTCG	136	T/C	0.08333333	121	KY568040
2512	Mam	F-CTTAAGCAGCAAAAGTTA R-GTTCCACTATCCACCACCCG	45	G/A	0.05555556	132	KY568041
3179	Mam	F-AGACTAGATTACAGAGGAGGCC R-ACCCTAGCCTTATGGGCTATGT	155	G/A	0.28571429	131	KY568042
3570	Mam	F-TAACTAACATATTTATTCTT R-ACCTATTGTGACTGCTTGGA	27	C/T	0.08333333	170	KY568043
3578	Mam	F-TGCTCAATTGTCTGGCCCACA R-GATCCTCTGGTCGGCTCCAA	140	C/T	0.1	151	KY568044
3847	Mam	F-GACAACATAGGGTCCGGT R-TCCCCTCTCAGCCTTCTCTT	60	A/T	0.05555556	121	KY568045
4040	Mam	F-GAACGTCATCCTCTTAGCTT R-TGCTGGCATAGAAAGTTT	44	C/T	0.27777778	115	KY568046
4481	Mam	F-TCTCCCTCATCTGGACCTCC R-TGACTCCCAGGAAAAAGCCAACA	165	C/T	0.0625	112	KY568047
4503	Mam	F-AGGGCTTGTTTACTGCTGCT R-CATCAATGTCAAAAAATATGC	146	T/G	0.11111111	107	KY568048
5192	Mam	F-GCTGGCAGCCAGCAAGGCCG R-TGCAAGTCCAGAGCCCTAAATA	39	A/G	0.05555556	168	KY568049
5269	Mam	F-ACCTGTCTGCTTCACCAAGAG R-GGGTTTTTACAGTATCAACT	127	G/A	0.05555556	116	KY568050
5494	Mam	F-CATTCCTGTCTGACATTCCACA R-AACTCTATCCTGGGAAAAAG	51	T/A	0.05555556	133	KY568051
5645	Mam	F-GTAAGTGACCACCGTAGAGA R-TGGCTTAGAGGAGAACCGGT	26	G/A	0.0625	114	KY568052
6308	Mam	F-GGAAGGGAAGGAGAGAGAAAAG R-ATATCTGATCCACCACCTTC	55	A/G	0.0625	149	KY568053
6554	Mam	F-CCTCCTGGGGCATCAAACAT R-GCTGCTGATCAACATGGTGG	104	G/C	0.14285714	119	KY568054
7029	Mam	F-TTCTTCAGTGTTTCCTGACG R-AGTTGGGAGCTGTTCATGCA	28	A/G	0.08333333	188	KY568055
7153	Mam	F-ATGCTTTGTGTGATCCAATATT R-GTGGCATGGTCACATTCAGG	56	A/T	0.05555556	148	KY568056
8164	Mam	F-CAGACCTGGGAGCTGTCAG R-TTGTGGAAGGGAGATTTCTGCC	173	T/G	0.07142857	145	KY568057

Locus ID	Locality	Primer (5' – 3')	Position	Alleles	Q frequency	Product size	GenBank accession number
8481	Mam	F-GCACTGCACTTCAAGAGGGA R-CTCTTGCAGAGGCTGAAAAT	133	T/A	0.05555556	134	KY568058
8571	Mam	F-GCGTGGGGATTCTGATACC R-CTGAGTTAGGCATGGGGAGA	40	A/C	0.11111111	167	KY568059
11138	Mam	F-TTTGCTGCCTCATCCCTCAG R-CAATAGCTACTTTGCCTCAGTT	163	G/A	0.05555556	108	KY568060
542	Jan	F-GGCAAAAGGTGGGGAAGGTA R-TTCCTGGACTCACATCTCTGC	162	A/G	0.08333333	131	KY568061
1730	Jan	F-AATATAGAAAAAACATTTAAG R-GCAGCCGAATTTCCAAATCCA	38	A/G	0.33333333	134	KY568062
3266	Jan	F-CGTGGTTGTCTTGTCCTCCA R-CTGTGACTCGGACCCTAAAA	138	C/T	0.2	155	KY568063
3784	Jan	F-ACCCTTGGGTGTAAACTGTC R-AGGCTCAGGCAAGGGAGATC	94	A/G	0.08333333	112	KY568064
4063	Jan	F-TACATAATACAAATCATACAC R-GCTGCTGCCTGGAAAGATTC	69	T/C	0.1	130	KY568065
4269	Jan	F-GGACCTCATAAAAACAAGAT R-CTAGTGGCAGCTCTCCCCT	59	C/T	0.1	137	KY568066
4490	Jan	F-GCTGCTGTGTTTTTCACTGTGG R-TCAAAGAGAGTTAGAGCATGTG	54	C/G	0.1	149	KY568067
5235	Jan	F-TGTGTGCTCTGGGTCTGAGCTT R-CTGTGGCAGCAGCTCTGG	109	A/T	0.08333333	104	KY568068
6049	Jan	F-CTGCCTGCCGTGATAGTTCA R-CCTCCTGCCTCCAGTCTTGTGT	105	G/A	0.1	116	KY568069
6189	Jan	F-CAGCCTGCCTTAATCACATTAT R-ACTTGACCTGAGGGGAGGAA	41	G/T	0.08333333	175	KY568070
6289	Jan	F-GGCCCCCCCCGGTTGAGGGGGT R-TCTCTCACCTCCACACCTCC	34	C/T	0.125	111	KY568071
6633	Jan	F-GATTGCATCCATCGCCAGCT R-GAGATATTTTGCCCAGTTTCTA	129	C/T	0.08333333	111	KY568072
7175	Jan	F-AGGAGTGTAGGGAGATATGA R-TGTCATGTCTGTCAGTGCCC	97	G/A	0.16666667	110	KY568073
7345	Jan	F-GTATCCGCTGTTGAGGCCAA R-ACATGAGTTTTGCTTTCAGG	97	C/A	0.16666667	101	KY568074
7468	Jan	F-TTTGGTAGATTTGCTTCTGACC R-ACCTGCTCCATCCTTGCTTC	31	T/C	0.1	125	KY568075
7517	Jan	F-GGACCACCTATCAAGCCCTA R-GCTAAGTATTCATGTGTTTA	131	C/T	0.2	145	KY568076
7648	Jan	F-TCCCTGCTGTCTACCCTGAG R-AAGTAAGTGGACTGGAATATTA	120	C/T	0.08333333	125	KY568077
7663	Jan	F-TCTTTTGATAGGTTTAGCATGC R-CATCTCTGCCTGAAGCCCAG	45	T/C	0.125	117	KY568078
7841	Jan	F-GGTGTAAACTGTCAGGGCCT R-CCCATTACAGGACCGGGGGT	112	G/A	0.1	123	KY568079
9166	Jan	F-CAGTATGGGAGGGTGCTGGCA R-TGGTCTGTGCTAGGATTCAT	23	C/A	0.125	138	KY568080
10240	Jan	F-CTTCCTAGTGAGAGAGATTTTC R-GCTTTCAACTACCTGAAGGA	139	C/T	0.1	147	KY568081

Locus ID	Locality	Primer (5' – 3')	Position	Alleles	Q frequency	Product size	GenBank accession number
11848	Jan	F-GTGGAACCAGAGGCTTTCGA R-CATACCCAGGAGCTGGAGGAGA	160	C/T	0.1	130	KY568082
1295	LM	F-TACTGGAAGGGGCTCCGGCAC R-TCTCTAGCCCTCCTGCTCTGCT	124	T/A	0.5	145	KY568083
1651	LM	F-AGTGAAAATAGAAGGATTAC R-GGAACAGTGATCATACTTCTCT	29	T/C	0.5	183	KY568084
6334	LM	F-ACACAATAGCCCCTTTGCAAG R-AGCAGTTTCAGCACTGGTGGCTGT	114	G/A	0.33333333	118	KY568085
6560	LM	F-ACACCAAGGTTGTATAGCC R-ATTAGATTACCTTACGGCAGC	101	A/G	0.41666667	105	KY568086
6967	LM	F-AGGTGCTTCGCTAGGTCTCCA R-AGAAAACGCTGCTGATCCCT	72	G/A	0.125	120	KY568087
10449	LM	F-AGGTCTAGCTCATCCTCCCG R-CAGTAAATGTCTCGGTGTCTAC	130	A/G	0.41666667	120	KY568088

Note: The 78 SNPs markers were chosen based on the following criteria: no linkage disequilibrium with other loci; SNP locus contains private alleles for each geographical location of *Melanosuchus niger* (Ana: Anavilhanas; Mam: Mamiraua; Jan: Janauaca; LM: lower Madeira); locus must be present in all populations; a minimum 4x depth coverage per allele; a lower limit for MAF (minor allele frequency) of 0.01 and 50% of individuals in population must have the locus. Position refers to a SNP site within the tag. Q frequency refers to frequency of less frequent allele. GenBank accession number refers to the less frequent allele of each locus. The SNP is located at the 3'extremity of one of the primers, shown in red.

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