Short Communication
Identifying sequence variation in cation channel sperm associated genes in Cape mountain zebra *(Equus zebra zebra)*

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Abstract
The Cape mountain zebra (*Equus zebra zebra*) has recovered from near extinction over more than eight decades. While their numbers have increased, populations remain isolated with low genetic diversity. With more than 75 new populations being founded and more than 4800 extant animals, conservation management strategies are being implemented to mitigate risk of losses in genetic diversity and reproductive fitness. One objective is to identify reproductive characteristics that may improve population growth. Cation channel sperm (CatSper) genes play an important role in hyperactivation of sperm during fertilization. Mutations in these genes lead to reduced fertility and even infertility. Ten male zebras were sampled from a group that were translocated in 2016 in order to found a new population. Single nucleotide polymorphisms (SNPs) were identified in three of the CatSper genes (1 - 3). Lack of variation was observed in all exons, with only four SNPs being identified in the intronic regions in close proximity to exons 1, 2, 7, 8, and 9 of CatSper 1. These results may contribute to the pre-identification of males for new founder populations to ensure population growth and viability, and may be a useful tool for selection against low-producing individuals.

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Cape mountain zebra (CMZ) *(Equus zebra zebra)* is a sub-species of mountain zebra that is distributed only in South Africa (Novellie *et al.*, 2002). These animals have recovered from a severe population bottleneck, which took place in the 1930s (Hrabar & Kerley, 2013), to a current estimated population size of more than 4800 (Birss *et al.*, 2018). Because of reported low genetic diversity (Moodley & Harley, 2005), a biodiversity management plan was developed that focused on strategies and actions to strengthen overall population performance, distribution and genetic diversity to ensure fitness and resilience of the meta-population within the natural distribution range (Birss *et al.*, 2018). Since the 1950s, the number of Cape mountain zebra has increased gradually through the founding of new populations to ensure continued population growth (Novellie *et al.*, 2002). To date, the overall population occurs in more than 75 localities, which include 30 national and provincial parks. As many as 90% of the founding CMZ were sourced from Mountain Zebra National Park and 50% of all populations were founded with fewer than the recommended number of founding animals (Moodley & Harley, 2005).

Low reproductive success in CMZ mares (foaling rate of 32%) has been reported in Mountain Zebra National Park (Penzhorn, 1985) and in De Hoop Nature Reserve (DHNR) (Birss, 2018, pers. comm.). In addition, abnormal sperm heads because of a weak head-neck junction have been identified in a CMZ stallion (Penzhorn & Van der Merwe, 1988). Whereas several genes control sperm motility, the calcium channel of sperm (CatSper) is studied most (Ren *et al.*, 2001). The CatSper protein family consists of specialized calcium (Ca^{2+}) channel proteins that are expressed exclusively in the sperm flagellum (Hildebrand *et al.*, 2010) and thus are directly involved in hyperactivation of the spermatozoa and penetration ability of the *zona pellucida* (Stauss *et al.,* 1995). The CatSper complex is reported to include four subunits

(CatSper 1 - 4) and three auxiliary subunits, namely CatSperβ, CatSperδ and CatSperγ (Navarro *et al.*, 2008; Wang *et al.*, 2009; Chung *et al.*, 2011). CatSper 1 - 4 are expressed in spermatozoa and are functional on the principal piece of the sperm tail (Qi *et al.*, 2007). This action is achieved through the use of Ca^{2+} ions, which control swimming behaviour through the ion pump action in the flagellum (Armon & Eisenbach, 2011). CatSper has been identified as a necessary component for reproductive success in mice (Ren *et al.*, 2001; Carlson *et al.*, 2003; Qi *et al.*, 2007), human beings (Avenarius *et al.*, 2009; Hildebrand *et al.*, 2010; Strünker *et al.*, 2011; Saha *et al.*, 2015), and horses (Loux *et al.*, 2013). Mutations leading to infertility have been reported in all four subunits of CatSper. In CatSper 1, two insertion mutations (c.539-540insT and c.948-949insATGGC) were reported that led to infertility in humans (Avenarius *et al.*, 2009). Mutations in the CatSper 2 gene also lead to low sperm counts in humans (Zhang *et al.*, 2009) and a copy number variation was identified that caused infertility (Luo *et al.*, 2019). CatSper 3 and 4 mutations in mice were shown to cause infertility (Jin *et al.*, 2007). Mutations that lie within the functional domain of CatSper 3 (c.193T>C) and CatSper 4 (c.247A>G, c.157T>C, c.992G>A) genes were identified in humans and are associated with asthenozoospermia (Visser *et al.*, 2011). The current study was undertaken to screen the CatSper 1 - 3 genes to determine nucleotide variations in CMZ as potential DNA markers associated with improved sperm motility. The genotype of an individual may serve as a criterion when selecting animals to be translocated to ensure population growth and viability.

Ethics submissions were approved by the University of the Free State Animal Ethics Committee (UFS-AED2017/0011) and Research Ethics and Scientific Committee of the National Zoological Garden, South African National Biodiversity Institute (NZG SANBI, NZG/RES/P17/19. The Department of Agriculture, Forestry and Fisheries of South Africa granted a permit under Section 20 of the Animal Diseases Act of 1984 (Ref: 12/11/1/1/8). Samples were collected under a Threatened or Protected Species Regulations Permit (No. 07507) through the Department of Environmental Affairs of South Africa.

Blood samples from 10 male CMZ were collected from DHNR. Two males were identified as foals, based on their size, presence of fluffy coat and deciduous teeth. Another two males were designated sub-adult because of the presence of undescended testicles. Six males were identified as adult stallions.

Reference sequences from horse *(Equus caballus)* from Ensembl were used to design the primers, namely CatSper 1 (ENSECAG00000024405), CatSper 2 (ENSECAG00000020759) and CatSper 3 (ENSECAG00000014744). The primers were designed in flanking regions of each exon (Table 1). DNA was extracted from the whole blood using the Zymo Quick-DNA™ Universal kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions for biofluid and cells. Extracted DNA was stored at -20 °C until further analysis. The DNA fragments were amplified using *Taq* DNA polymerase Master Mix RED (Ampliqon A/S, Odense M, Denmark) in 15 µℓ reactions, which included forward and reverse primers (0.5 µM), 50 ng of genomic template and GC enhancer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The reactions were run under these conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 seconds, annealing for 30 seconds, followed by elongation at 72 °C for 30 seconds with a final elongation step of 72 °C for 10 minutes. Polymerase chain reactions were carried out in a T100™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA). The amplified fragments were purified with Exonuclease I (Thermo Fisher Scientific Inc.) and FastAP thermosensitive alkaline phosphatase (Thermo Fisher Scientific Inc.) in a thermal cycler at 37 °C for 15 minutes, followed by 85 °C for 15 minutes. Next, the fragments were used as a template for sequencing using the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, California, USA) according to the manufacturer's instructions. Briefly, 1 µℓ of BigDye™ Terminator v3.1 ready reaction mix, 3.2 pmol of either the forward or reverse primer, and 1 x BigDye™ Terminator v1.1 & v3.1, 5X sequencing buffer, were prepared in a mastermix with 5 µℓ of the amplified PCR product made up with nuclease-free water to 10 µℓ. Sequencing was conducted in a thermocycler using these parameters: denaturation at 94 °C for 2 minutes, 40 cycles of 85 °C for 10 seconds, 53 °C for 10 seconds and 60 °C for 4 minutes. The resulting reaction was then purified using the BigDye[®] Xterminator™ sequencing purification kit, as recommended by the manufacturer. The DNA products were sequenced on ABI 3130 genetic analysis (Applied Biosystems Inc.). The resulting outputs were analysed with sequencing analysis software v6.0 (Applied Biosystems Inc.).

The sequence files were inspected visually and the chromatograms were edited and assembled (forward and reverse sequences) using Geneious[®] v10.2.6 software (Kearse *et al.*, 2012) and the default parameters. Low-quality sections at the ends of the sequences were trimmed manually. A multiple sequence alignment was carried out for all ten samples. Horse sequences for each of CatSper genes 1 - 3 were used as a reference. The resulting alignment was inspected visually for sequence variants such as insertions, deletions and base-pair variations. Single nucleotide polymorphisms between the horse reference sequence and zebra were not considered here.

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Table 1 Primers used in polymerase chain reaction to amplify Cape mountain zebra CatSper genes 1 - 3

Primer Name	Forward sequence	Reverse sequence	Annealing temperature (°C)
CAT1_1	AACCCTCGATGGCTGGAAAG	GGACGGTGAGCAAAGACTCA	62
CAT1_2	TCAGAAGCGCAAAGGTGAGT	GGCTCCCTGGTTCTTACCAC	60
CAT1_3	GCTGCACACCTTGTACCTCT	CAGTCCCATCCCTTGAGCAG	60
CAT1_4	CCTCTGCATCTACGTGGTGG	GGGGGTTGTTCGAACTGTGA	53
CAT1_5	CTTTACCCTGCTCACCCTGG	TTCACCCGGAAAGTCAGGTG	60
CAT2_1	TGAGTCTTAGTACAATGTGT	ATCCTACTCCAGGAGACA	55
CAT2_2	TCTGATCATTCTCTATCATTC	TGTTCCATTCCTGTATCTC	55
CAT2_3	TCTGAGAGGTTTAGATCTC	GAGCTGGGGAATTCTAAC	55
CAT2_4	CTACACTTCTGCTTCAGTAT	GTTTCATAGAAGGTGCTTGTA	55
CAT2_5	CCATATCTTGTAAATGTA	GTATGGATTTAGGGCAAT	55
CAT3_1	GCAGACTTTAGTTGCTAC	CATAGGGTCTGGACTATTC	55
CAT3_2	GCTCTGCAGCTTGATCTG	AGTCAGACACACCTTTCA	55
CAT3_3	GGCATGGCACTGGATACT	CAGCCCTGATTGTCCATC	55
CAT3_4	GGTGTCATCTTCTTATCATTGC	ACTGATGGTCTGGAGTCC	55

Here, for the first time the authors report sequence analysis of CatSper 1–3 in CMZ to identify possible nucleotide variations associated with sperm motility. The sequence data covered a range of exons (Table 2), which provided complete and, in some cases, partial coverage for some of the exons. Partial fragments were obtained because of the selected primer regions or because the target regions were too long for the sequencing method.

The role of CatSper genes in sperm motility is widely reported and the products of these genes are recognized as the most important calcium channels required for fertility in mammals (Singh & Rajender, 2014). Single nucleotide polymorphisms in the CatSper genes associated with sperm motility have been identified in Vrindavani cattle (Sivakumar *et al.*, 2017) and mice (Qi *et al.*, 2007). Knock-outs of the CatSper genes may cause infertility in humans without affecting normal sperm production (Singh & Rajender, 2014).

This study revealed that CMZ males have exons that are highly conserved within the sample with an absence of SNPs in exons of CatSper genes 1–3. The absence of SNPs in this study may be attributed to the number of animals and to the low genetic diversity of the population (Moodley & Harley 2005; Kotzé *et al.*, 2019). Since only a number of animals from a single population were used here, it would be useful to compare variation between the isolated populations of CMZ. Use of additional populations will establish how well conserved these genes are within the subspecies. A lack of SNPs in CatSper may mean that polymorphisms in coding regions of other genes may be responsible for changes in sperm motility in CMZ. Alternatively, this absence of SNPs may indicate that additional ecological reasons should be considered for the slow increase in population growth of CMZ. High grass abundance has been associated with higher population growth rates and zebra density and less skewed adult sex ratios (Lea *et al.*, 2016). De Hoop Nature Reserve had a large proportion of unsuitable habitat, with most CMZ grazing on only 30% of the total area of the reserve (Smith *et al.*, 2007).

Here, portions of introns were also sequenced, and the authors report four SNPs that were identified in the intronic regions of 1, 7, and 9 of CatSper 1 (Figure 1). These are G1547A, which is 89 bp downstream from exon 1; G2241A, which is located 126 bp upstream from exon 2; C4675T, which is found 43 bp downstream from exon 7, and G5270A, which is located 206 bp downstream from exon 9. Studies have shown that SNPs within the introns of genes play a role in mRNA expression (Nott *et al.*, 2003; Wang *et al.*, 2011; Zhang *et al.*, 2014) and determine the phenotypic expression of certain traits, such as eye colour in humans (Sturm *et al.*, 2008). Thus, these SNPs may have a role in the expression of the CatSper 1 protein in the mid piece of the sperm tail. Additional analysis is required to determine whether the SNPs in these regions influence the expression of the CatSper 1 gene.

Table 2 Coverage of exons obtained from primers that were designed in this study

| Gene	Fragment	Exon covered	Partial/Full	Coverage (%)
CatSper 1	CAT1_1	Exon 1	Partial	88
	CAT1_2	Exon 2	Full	100
	CAT1_3	Exon 3	Partial	41
	CAT1_3	Exon 4	Full	100
	CAT1_5	Exon 7	Full	100
	CAT1_5	Exon 8	Full	100
CatSper 2	CAT2_1	Exon 1	Full	100
	CAT2_1	Exon 2	Full	100
	CAT2_1	Exon 3	Full	100
	CAT2_2	Exon 4	Full	100
	CAT2_2	Exon 5	Full	100
	CAT2_2	Exon 6	Full	100
	CAT2_3	Exon 7	Full	100
	CAT2_3	Exon 8	Full	100
	CAT2_5	Exon 9	Full	100
	CAT2_5	Exon 10	Full	100
CatSper 3	CAT3_1	Exon 2	Full	100
	CAT3_2	Exon 3	Full	100
	CAT3_3	Exon 4	Full	100
	CAT3_3	Exon 5	Full	100
	CAT3_3	Exon 6	Full	100
	CAT3_4	Exon 7	Partial	59
	CAT3_4	Exon 8	Full	100
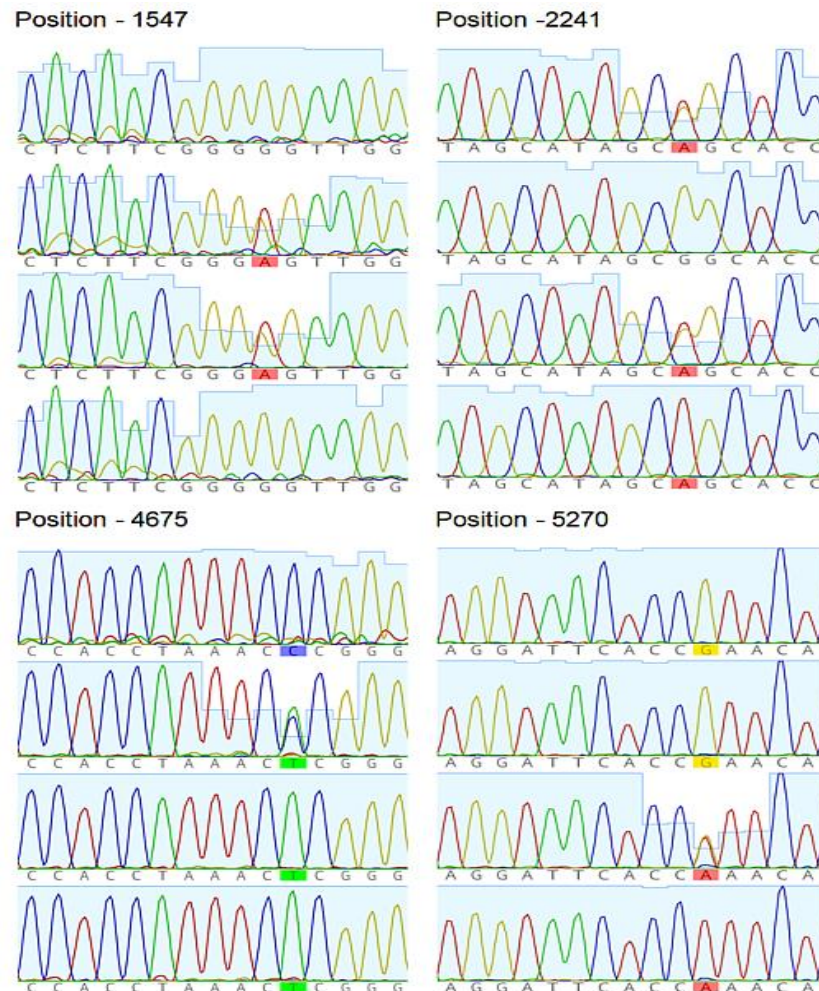
Figure 1 Representative sequence chromatograms showing positions of single nucleotide polymorphisms identified in the intronic regions of CatSper 1
These positions are a) G1547A, b) G2241A, c) C4675T and d) G5270A

Future unforeseen environmental stochasticity may necessitate the use of artificial fertilization techniques in CMZ to increase the reproductive output in key reserves to maintain genetic diversity and population viability. Identifying SNP variations within the introns and exons of genes associated with fertility may provide a criterion for selecting suitable candidates. Further studies on a larger sample set could include additional genes such as glutamine-rich protein 2 and A-kinase anchoring protein 4, which have been reported to identify a loss of sperm function (Shen *et al.*, 2019) and reduced sperm motility (Moretti *et al.*, 2007). After functional correlations have been established, structural changes in the protein could be better understood. In future comparative studies between zebra species that characterize sperm and other physiological parameters may be useful to diagnose potential defects in stallions, should semen samples become available.

Authors Contributions
        RMS conceptualized the research question, collected the data for this research, made a leading contribution to original manuscript, and carried out the statistical analysis. AK provided the platform for research and contributed to

reviewing the manuscript. JPG reviewed the manuscript and DD also conceptualized the research question, carried out analysis, and contributed to the original manuscript

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