Short communication

Hiding in plain sight: evidence of hybridization between Cape mountain zebra (*Equus zebra zebra*) and plains zebra (*Equus quagga burchelli*)

Desiré L. Dalton[1,2]*, David Zimmermann[3], Clearance Mnisi[1], Megan Taplin[4], Peter Novellie[3], Halzska Hrabar[5] & Antoinette Kotzé[1,2]

[1]*National Zoological Gardens of South Africa, P.O. Box 754, Pretoria, 0001 South Africa*

[2]*Genetics Department, University of the Free State, P.O. Box 339, Bloemfontein, 9300 South Africa*

[3]*Veterinary Wildlife Services, South African National Parks, P.O. Box 110040, Hadison Park, Kimberley, 8306 South Africa*

[4]*Mountain Zebra National Park, South African National Parks, Private Bag X66, Cradock, 5880 South Africa*

[5]*Centre for African Conservation Ecology, Department of Zoology, Nelson Mandela Metropolitan University, P.O. Box 77000, Port Elizabeth, 6031 South Africa*

Received 13 June 2016. To authors for revision 20 July 2016.
Accepted 10 October 2016

INTRODUCTION

Historically, Cape mountain zebras (*Equus zebra zebra*) were widely distributed along mountain ranges in the Eastern and Western Cape provinces of South Africa (Boshoff, Landman & Kerley, 2015). By the 1930s, excessive hunting and habitat loss resulted in a reduction in Cape mountain zebra numbers with populations being confined to only five localities. Two of these subpopulations subsequently became extinct. Three relic populations currently exist; the population in the Cradock district, was formally protected in 1937 by the proclamation of the Mountain Zebra National Park (MZNP; Lloyd 1984). The two other populations in the Kammanassie and Gamka Mountains, have been protected since 1923 and 1971, respectively. Cape mountain zebra numbers increased steadily from their critical status of fewer than 80 individuals in the 1950s, to an estimated minimum of 4791 individuals by 2015 (Hrabar & Kerley, 2015). Plains zebra (*Equus quagga burchelli*) were subsequently introduced, in sympatry with Cape mountain zebra into four formally protected areas, including the MZNP in 1999 and Karoo National Park in 1998. Until recently no cases of hybridization between plains zebra and Cape mountain zebra were known. Hybridization was not of great concern as a threat to Cape mountain zebra populations as fertile hybrids were thought to be unlikely, due to the relatively large difference in the number of chromosomal pairs between the two species (44 *versus* 32 in plains zebra and Cape mountain zebra, respectively; Ryder, Epel & Benirschke, 1978; Cordingley *et al*., 2009; Hrabar & Kerley, 2013). By 2013, the plains zebra population had increased substantially in the MZNP (estimated at 769 Cape mountain zebra and 124 plains zebra by aerial census (unpublished aerial census data, 2013) and were potentially competing with Cape mountain zebra for resources. A decision was thus taken to remove the plains zebra. This intervention resulted in a disruption in the social structure, and some of the small, fragmented groups or plains zebra individuals joined Cape mountain zebra herds. In addition, conservation officials observed 'Cape mountain zebra' with plains zebra characteristics. These included slight shadow striping, stripes extending all the way down to the ventral midline of the chest and abdomen, and, although they did have the reddish muzzle of mountain zebra, they did not have the characteristic mountain zebra gridiron pattern on their rumps (Fig. 1), but rather had absent or distorted patterns on the rump. They exhibited the distinct dewlap of the Cape mountain zebra and ear shapes were similar to plains zebra. This raised concerns of possible hybridization between the two species. Here, we report on a molecular evaluation using maternal, paternal and biparental markers to identify suspected hybrid Cape mountain and plains zebra in MZNP and Karoo National Park, South Africa.

MATERIALS AND METHODS

Ethical approval for this project was obtained through the Research and Ethics Scientific Committee of the National Zoological Gardens of South Africa number: P13/10. Blood samples were collected in ethylenediamine tetraacetic acid (EDTA) tubes from 101 animals located in the MZNP, of which four stallions were suspected of

*To whom correspondence should be addressed.
E-mail: desire@nzg.ac.za

ISSN 2410-7220 [Print], ISSN 2410-8200 [Online] — DOI: http://dx.doi.org/10.3957/056.047.0059

Fig. 1. Representative phenotypes of Cape mountain zebra (top), plains zebra (middle) and their interspecific hybrid (bottom).

being hybrids, this based on their morphological appearance (332/14 and 335/14) or association within a family group (334/14 and 341/14). In addition, ten samples were collected from the Karoo National Park. A combination of etorphine hydrochloride (M99, Novartis), azaperone (Stresnil, Janssen-Cilag) and hyaluronidase (Hyalase, Kyron Laboratories) was used to immobilize the animals. Semen sample to examine sperm quality and quantity, of the suspect hybrid stallions, could not be collected. Reference samples of Cape mountain zebra had previously been collected from Mountain Zebra National Park ($n = 7$) and private game farms ($n = 88$) and reference samples of plains zebra were collected from private game farms ($n = 26$) that have no history of co-occurance of the two equids.

A total of 16 microsatellites markers developed for Grevy's zebra *E. grevyi* (Ito, Hayano, Langenhorst, Sakamoto & Inoue-Murayama, 2013) were

used to genotype all individuals using the reported amplification conditions. PCR products were pooled together in four multiplexes and were run against Genescan™ 500 LIZ™ (Applied Biosystems, Inc.) internal size standard on an ABI 3130 Genetic Analyzer (Applied Biosystems, Inc.). Samples were genotyped using GeneMapper v. 4.0. The genetic relationships between populations and individual assignments of pure plains zebra, Cape mountain zebra and putative hybrids was explored in two different ways. First, we identified private alleles by determing which alleles were present in one species but not the other. Secondly, purity was inferred *via* a Bayesian clustering analysis using the statistical programme STRUCTURE version 2.3.3 (Pritchard, Stephens & Donnelly, 2000). Assessments were conducted with the USEPOPINFO option active and without prior population information (option USEPOPINFO=0). STRUCTURE was run for 5 replicates from K = 1–4, with a run-length of 500 000 repetitions of Markov chain Monte Carlo, following the burn-in period of 20 000 iterations. The four values for the estimated ln(Pr(X|K)) were averaged, from which the posterior probabilities were calculated. The K with the greatest increase in posterior probability (Evanno, Regnaut & Goudet, 2005) was identified as the optimum number of sub-populations using STRUCTURE HARVESTER (Earl & von Holdt, 2012). From the selected K value, we assessed the average proportion of membership (QI) to the inferred cluster of the sampled populations.

A subset of samples was characterized to determine maternity and paternity through amplification of a 1.2 kb mitochondrial D-Loop region using the protocol reported in Moodley & Harley (2005) and a 390 bp fragment of Y chromosome DNA (Cordingley *et al*., 2009). Resulting sequence chromatograms were edited in the Chromas program embedded in MEGA6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2011) prior to performing a BLAST nucleotide search (www.ncbi.nm.nih.gov/blast). A maximum likelihood phylogenetic tree model test was performed in MEGA6 to determine the best possible nucleotide substitution model fit for the sequence data. Maximum likelihood parameters were evaluated and scored. A total of 24 different models was assessed and the Hasegawa-Kishino-Yano model (Hasegawa, Kishino & Yano, 1985) was selected as it had the highest maximum likelihood parameter score (90%), making it the best possible model fit to accurately represent our sequence data. Nodal support for the Likelihood (ML) tree was assessed through 10 000 non-parametric bootstrap replications.

RESULTS

A total of 98 alleles were detected, with 42 specific to plains zebra, 23 to Cape mountain zebra; 31 were shared (Table S1 in online supplement). Posterior probabilities (*Ln*) using Bayesian admixture analysis indicated two distinct clusters (Fig. 2). The average proportion of membership for both pure populations was $q_i > 0.992$. The criterion of $q_i > 0.90$ suggested by Barilani *et al*. (2007) can be used to identify individuals as either pure or hybrid, and is used extensively in hybridization studies in various species (Oliveira, Godinho, Randi, Ferrand & Alves, 2008; Quintela, Thulin & Höglund, 2010). Of the four suspected hybrids, 332/14 ($q_i = 0.428$) and 335/14 ($q_i = 0.391$) were genetically identified as hybrids, whereas 334/14 was identified as pure Cape mountain zebra and 341/14 as a pure plains zebra. Phylogenetic analysis identified distinct clades for plains and Cape mountain zebra with 98% bootstrap support between 37 mtDNA sequences generated here (GenBank accession numbers KX906976–KX907012) and seven previously published sequences obtained from GenBank (Fig. S1 in online supplement). The two hybrid animals (332/14 and 335/14) identified *via* genetic analysis clustered with Cape mountain zebra based on mitochondrial DNA. Y chromosome amplification conducted on a subset of samples ($n = 10$) was successful for five plains zebra, four Cape mountain zebra and the hybrid 332/14 (Table S2 in online supplement). Based on the identification of a single nucleotide polymorphism (SNP), the Y chromosome sequences of 332/14 are characteristic of plains zebra, and the paternal lineage of hybrid 332/14 was identified as plains zebra. Thus the genetic data (mitochondrial and Y chromosome sequences) provides unequivocal evidence of hybridization, in this particular instance (332/14) involving a cross between a Cape mountain zebra mare and a plains zebra stallion.

DISCUSSION

Hybridization can occur due to poor habitat and/or habitat modification, human-mediated introductions, small populations, skewed sex ratios and low mate availability (Jansson, Thulin & Pehrson, 2007). Molecular genetic tools assist in identifying hybridization with greater accuracy and have

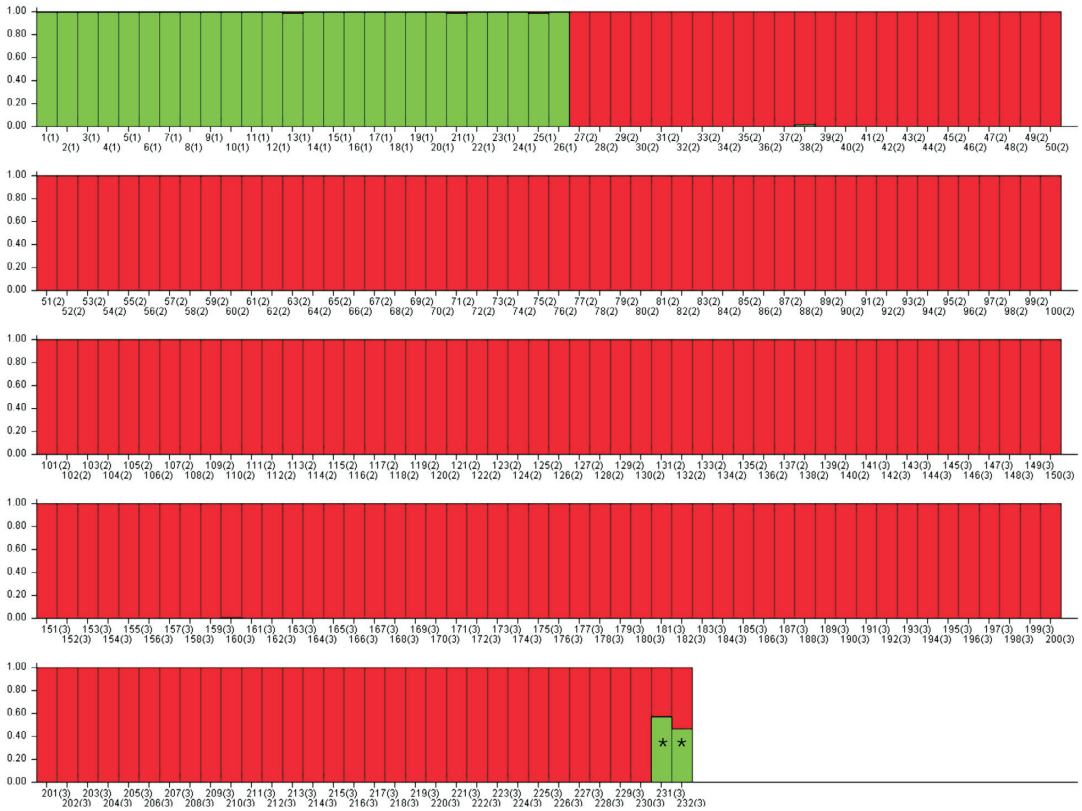

Fig. 2. Output from Likelihood histograms from STRUCTURE analysis for K = 2 averaged results of five runs. Each individual is represented by a single vertical line, with lengths proportional to the estimated membership in each cluster. Admixed genotypes (indicated with a *), at threshold $q_I = 0.90$ are evident.

proven to be useful in identifying unique management units. In this investigation, genetic analysis confirmed hybridization of the two (332/14 and 335/14) stallions that had the appearance of suspected hybrids. The young stallion (341/14), although associated with a Cape mountain zebra family group, but morphologically indistinct from a plains zebra and suspected to be a potential hybrid, was genetically indistinct from a plains zebra. The subadult mare (334/14) associated with suspected hybrid stallion 332/14 (and therefore flagged as a potential offspring of a suspect stallion) was found to be morphologically and genetically characteristic of Cape mountain zebra. These findings provide insights as to the social dynamics resulting in hybridization. Cordingley *et al*. (2009) reported on hybridization between Grevy's and plains zebra and identified all hybrids with maternal haplotypes of plains zebra. They hypothesized that hybridization may have occured due to a male-biased sex ratio resulting in Grevy's zebra males seeking mating opportunities with plains zebra females. In the present study, the identified hybrid individuals were adult zebra (4–5 years old), indicating that hybridization had taken place at least 5–6 years prior to this investigation.

When plains zebra were introduced to MZNP in May 1999, the initial 14 zebra included only one adult stallion. In November that year a further three stallions, from a different source, were introduced. In 2000, the plains zebra population was further supplemented with three stallions and three mares. The small founder population with a relatively high number of stallions may have led to a low mate availability. Adult plains zebra stallions (313 kg) are substantially larger than Cape mountain zebra stallions (235 kg). It is hypothesized that plains zebra stallions who did not have a cohesive family group may have ousted Cape mountain zebra herd stallions and taken over family groups, as was seen with the young stallion (341/14) who associated with a Cape mountain zebra family group.

Analysis of the maternally and paternally inherited markers in this study indicate that at least one hybrid displayed Cape mountain zebra mitochondrial lineage and plains zebra Y chromosome lineage, indicating that mating occurred between female Cape mountain zebra and male plains zebra.

There is a substantial difference in karyotype between Cape mountain zebra (2n = 32) and plains zebra (2n = 44) (Heinrich, 1970). It has been reported that hybrids between equid species with different chromosome numbers are considered to be infertile (Ryder *et al*. 1978; Cordingley *et al*., 2009; Hrabar & Kerley, 2013). However, more recent evidence shows that differences in chromosome number does not constitute an absolute barrier; for example, Cordingley *et al*. (2009) observed fertile hybrids between plains zebra and Grevy's zebra (2n = 46) and Jónsson *et al*. (2014) reported evidence for gene flow involving three contemporary equine species. In this study, only F1 hybrids were detected, which may indicate that hybrids are infertile. In addition, an analysis of 101 Cape mountain zebra in MZNP only identified two hybrid individuals, further supporting the hypothesis of infertility in this case. However, a reproductive assessment of the Cape mountain and plains zebra hybrids should be done in future. Thus, the major detrimental effect of hybridization (Allendorf, Leary, Spruell & Wenburg, 2001) in this case is wasted reproductive effort rather than genetic mixing. Monitoring for potential hybrids at MZNP is being conducted; however, as the removal of the plains zebra had already been initiated, the long-term negative consequences to the MZNP population may be limited. The Kammanassie population is also potentially under threat, as no fences exist between the reserve and a neighbouring farm which has plains zebra (Hrabar & Kerley, 2015). The Karoo National Park, with the second largest population, is currently still exposed to plains zebra but the removal of all plains zebra has been initiated. At present the only relic Cape mountain zebra population not threatened with hybridization is the population at Gamka Nature Reserve. It is recommended that plains zebra be removed from all locations where both species are managed sympatrically in order to prevent hybridization. The removals should ideally be done in one operation in order to avoid fragmentation of breeding herds, which may increase the risk of hybridization.

We would like to thank Piet Heymans for the photograph of the plains zebra, and Khosi Mathebula of SANParks Veterinary Wildlife Services for assistance with collection and processing of samples.

REFERENCES

Allendorf, F.W., Leary, R.F., Spruell, P. & Wenburg, J.K. (2001) The problems with hybrids: setting conservation guidelines. *Trends in Ecology and Evolution*, 16, 613–622.

Barilani, M., Bernard-Laurent, A., Mucci, N., Tabarroni, C., Kark, S., Garrido, J.A.P. & Randi, E. (2007). Hybridisation with introduced chukars (*Alectoris chukar*) threatens the gene pool integrity of native rock (*A. graeca*) and red legged (*A. rufa*) partridge populations. *Biological Conservation,* 137, 57–69.

Beja-Pereira, A., England, P.R., Ferrand, N., Jordan, S., Bakhiet, A.O., Abdalla, M.A., Mashkour, M., Jordana, J., Taberlet, P. & Luikart, G. (2004). African origins of the domestic donkey. *Science* 304, 1781–1781.

Boshoff, A.F., Landman, M. & Kerley, G.I.H. (2016). Filling the gaps on the maps: historical distribution patterns of some larger mammals in part of southern Africa. *Transactions of the Royal Society of South Africa*, 71, 23–87.

Cordingley, J.E., Sundaresan, S.R., Fischhoff, I.R., Shapiro, B., Ruskey, J. & Rubenstein, D.I. (2009). Is the endangered Grevy's zebra threatened by hybridisation. *Animal Conservation,* 12, 505–513.

Earl, D.A. & vonholdt, B.M. (2012). Structure harvester: a website and program for visualizing structure output and implementing the Evvano method. *Conservation Genetic Resources*, 4, 359–361.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software Structure: a simulation study. *Molecular Ecology*, 14, 2611–2620.

Heinrich, I.G. (1970). Karyological studies on southern African Perissodactyla. *Koedoe* 13: 51–108.

Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating the human–ape split by a molecular clock of mitochondrial DNA. *Molecular Evolution,* 22, 160–174.

Hrabar, H. & Kerley, G.I.H. (2013). Conservation goals for the Cape mountain zebra *Equus zebra* zebra security in numbers? *Oryx* 47, 403–409.

Hrabar, H. & Kerley, G.I.H. (2015). Cape mountain zebra 2014/15 status report. Report no. 63, Centre for African Conservation Ecology (pp. 3–26). Port Elizabeth, South Africa: Nelson Mandela Metropolitan University.

Ito, H., Hayano, A., Langenhorst, T., Sakamoto, H. & Inoue-Murayama M. (2013). Using next generation sequencing to develop microsatellite markers for the endangered Grevy's zebra (*Equus grevyi*). *Conservation Genetic Resources*, 5, 507–510.

Jansson, G., Thulin, C.G. & Pehrson, A. (2007). Factors related to the occurrence of hybrids between brown hares (*Lepus europaeus)* and mountain hares (*L. timidus*) in Sweden. *Ecology,* 30, 709–715.

Jónsson, H., Schubert, M., Seguin-Orlando, A., Ginolhac, A., Petersen, L., Fumagalli, M., Albrechtsen, A., Petersen, B., Korneliussen, T.S., Vilstrup, J.T. &

Lear, T. (2014). Speciation with gene flow in equids despite extensive chromosomal plasticity. *Proceedings of the National Academy of Sciences*, 111, 18655–18660.

Lloyd, P.H. (1984). The Cape mountain zebra 1984. *African Wildlife,* 38, 144–149.

Moehlman, P.D. (Ed.). (2002). Equids: Zebras, Asses, and Horses: Status Survey and Conservation Action Plan. Gland, Switzerland, and Cambridge: IUCN/SCC Equid Specialist Group, IUCN (The World Conservation Union)

Moodley, Y. & Harley, E.H. (2005). Population structuring in mountain zebras (*Equus zebra*): the molecular consequences of divergent demographic histories. *Conservation Genetics,* 6, 953–968.

Oakenfull, E.A., Lim, H.N. & Ryder, O.A. (2000). A survey of equid mitochondrial DNA: implications for the evolution, genetic diversity and conservation of *Equus*. *Conservation Genetics*, 1, 341–355.

Oliveira, R., Godinho, R., Randi, E., Ferrand, N. & Alves, P. C. (2008). Molecular analysis of hybridisation between wild and domestic cats (*Felis silvestris*) in Portugal: implications for conservation. *Conservation Genetics*, 9, 1–11.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Quintela, M., Thulin, C. G. & Höglund, J. (2010). Detecting hybridization between willow grouse (*Lagopus lagopus*) and rock ptarmigan (*L. muta*) in Central Sweden through Bayesian admixture analyses and mtDNA screening. *Conservation Genetics*, 11, 557–569.

Ryder, O.A., Epel, N.C. & Benirschke, K. (1978) Chromosome banding studies of the Equidae. *Cytogenetics and Cell Genetics*, 20, 332–350.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution,* 30, 2725–2729.

van Wyk, A.M., Kotzè, A., Randi, E. & Dalton, D.L. (2013). A hybrid dilemma: a molecular investigation of South African bontebok (*Damaliscus pygargus pygargus*) and blesbok (*Damaliscus pygargus phillipsi*). *Conservation Genetics,* 14, 589–599.

Vilstrup, J.T., Seguin-Orlando, A., Stiller, M., Ginolhac, A., Raghavan, M., Nielsen, S.C., Weinstock, J., Froese, D., Vasiliev, S.K., Ovodov, N.D. & Clary, J. (2013). Mitochondrial phylogenomics of modern and ancient equids. *PLOS ONE*, 8, p.e55950.

Responsible Editor: G.I.H. Kerley