Genetic variation within and among three ostrich breeds, estimated by using microsatellite markers

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Abstract

Genetic diversity within and among three ostrich populations was investigated to corroborate whether these populations can be classified genetically as three different breeds. The DNA of South African Black (SAB, n = 30), Zimbabwean Blue (ZB, n = 32) and Kenyan Redneck (KR, n = 17) birds was assessed for genetic differences using 19 microsatellite loci. The number of alleles, as well as observed and expected heterozygosity of alleles, was determined. Genetic differentiation was measured using the F-statistic (F_{ST}) and Nei's genetic distance. Significant differences were observed among the three breeds. The SAB and ZB (F_{ST} = 0.10 and Nei = 0.49) were genetically more similar, whereas the genetic distance between the KR and ZB breeds was the greatest (F_{ST}= 0.13 and Nei = 0.61). The SAB strain exhibited the greatest observed heterozygosity (H_o = 0.72) within its population while the ZB and the KR exhibited lower levels of heterozygosity (H_o = 0.68). Based on these results, it was suggested that crossbreeding between these breeds would lead to heterosis in commercial ostrich enterprises.

Keywords: Breed, genetic differentiation, heterosis, heterozygosity
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Introduction

The three ostrich breeds that currently comprise the genetic pool of the South African ostrich industry are the South African Black (SAB), Zimbabwean Blue (ZB) and the Kenyan Redneck (KR). The SAB is a hybrid breed, which resulted from crossing the Northern African ostrich *(Struthio camelus camelus)* with the Southern African ostrich *(S. c. australis)* to improve feather production in the early 1900s (Deurden, 1913). The ZB (125 kg) and KR (135 kg) are reported to achieve heavier live weights than the SAB (115 kg; Jarvis, 1998), with the ZB exhibiting a lower reproductive performance than the SAB (Brand *et al.*, 2005; Cloete *et al.*, 2008b). These breeds have been crossed haphazardly by the international ostrich industry without proper breeding goals, and no scientific data are available upon which to base crossbreeding decisions (Petite & Davis, 1999).

The South African ostrich industry is known to experience reproduction and chick survival problems (Cloete *et al.*, 2001). Reproduction and survival traits can be improved through the exploitation of non-additive genetic variation, expressed through hybrid vigour (heterosis) from crossbreeding. Heterosis can be obtained only if the breeds that are crossed are genetically distinct from each other. Therefore it is important to confirm this genetic distinction among the three breeds, where a breed can be defined as a group of animals within a species that have a common origin and certain physical characteristics that are distinguishable (Dalton, 1981). Different physical characteristics indicative of genetic differentiation have been observed among these three subspecies (Jarvis, 1998). However, the extent to which these phenotypic differences are the result of environmental influences or genetic effects has not been confirmed.

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Genetic differentiation between subpopulations can be quantified by the use of molecular markers, such as microsatellites. Microsatellites have commonly been applied in genetic diversity studies in poultry and livestock (Li *et al*., 2004; Zhou *et al*., 2005; Muchadeyi, 2007; Vicente *et al*., 2008; Mtileni *et al.*, 2011). Various molecular markers have specifically been used to investigate genetic diversity between ostrich subpopulations. These markers include restricted fragment length polymorphisms (RFLP) (Freitag & Robinson, 1993), minisatellite loci (Kawka *et al.*, 2007) and microsatellite loci (Kumari & Kemp, 1998; Kawka *et al.*, 2007). Genetic differences have been found between the KR and the Somalian Blue ostrich (Kumari & Kemp, 1998) and among the SAB, ZB and KR (Kawka *et al.*, 2007) using RFLP markers and microsatellite loci respectively. These studies had small population sizes which were assessed with as few as five microsatellite loci. It is therefore necessary to confirm these results using a larger sample size and more microsatellite loci. Microsatellites have also been used to construct a preliminary genetic linkage map of the ostrich (Huang *et al.*, 2008). This map can be of benefit for future identification of chromosomal regions affecting quantitative traits such as growth, reproduction and disease susceptibility.

Genetic differentiation among and within subpopulations can be measured by the F statistic (F_{ST}) (Hartl & Clark, 1997; Holsinger & Weir, 2009). The F statistic is directly related to the variance in the allele frequency among populations and to the degree of resemblance among individuals within populations (Holsinger & Weir, 2009).

Crossbreeding of the SAB with the ZB and KR may therefore result in heterosis and consequently the improvement of traits with economic importance in commercial production systems. The aim of this study was thus to determine whether there are significant differences among and within the three ostrich breeds that are currently available in South Africa. The results from the study could assist in finding the most suitable combination of these breeds to attain maximal levels of heterosis.

Materials and Methods

Blood samples were collected from the breeding flock (n = 188 breeding pairs) at Oudtshoorn Research Farm during 2007 and stored at 4 °C (Essa *et al.*, 2005), whereafter they were transported to Elsenburg DNA lab and stored at -18 °C for long-term use. Ethical clearance had been obtained from the Departmental Ethics Committee for Research on Animals (DECRA), under the DECRA reference number R11/37. In 2008, frozen blood samples were randomly selected from mature males and females in the breeding flock, comprising samples of 31 SAB birds and of 35 ZB birds. In contrast, only 17 frozen blood samples were available for the KR, representing the entire genetic resource for that breed at Oudtshoorn Research Farm. The SAB resource population was developed through the donation of 76 SAB breeder birds by 61 local producers in 1964. In the 1990s, more SAB birds were added to the flock and they were divided into two lines, namely the "commercial" and the feather strain (Bunter, 2002). According to Bunter, no evidence of heterosis was manifest in the crosses between these lines and they were subsequently treated as a single SAB genetic resource population. In 2003, 55 ZB breeding birds were added to the flock after being obtained from two local producers (Cloete *et al.*, 2008b). These birds originated from the Bulawayo and Harare districts of Zimbabwe. Possible genetic relationships among the base population of ZB birds as well as their exact ages were unknown, although their progeny were pedigreed. Nineteen KR birds (13 males and six females) of known ancestry were introduced to the flock. Their known pedigrees assisted in choosing birds that were not related for at least the last generation to represent this strain.

Birds were bled from the wing vein to collect at least 2 mL of blood in Becton-Dickinson (BD) Vacutainer™ K2EDTA tubes. The blood samples were stored for long-term use at -18 °C and thawed at room temperature for DNA purification. DNA was purified using the Proteinase K digestion standard phenol/chloroform/isoamylyl alcohol extraction procedures and absolute ethanol precipitation according to procedures, as described by Sambrook & Russell (2001).

The DNA was quantified using spectrophotometry and agarose gel electrophoresis. Polymerase chain reaction (PCR) was performed in a final volume of 5 µL consisting of 20 ng of template DNA, 0.4 µM or 0.6 µM of each primer, with KAPA 2G Fast Hotstart Readymix (KAPA Biosystems™). Primers were labelled with PET®, 6-FAM™, NED™ and VIC® fluorescent dye supplied by Applied Biosystems®. The PCR conditions for the KAPA 2G were denaturation at 95 °C for three minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 72 °C for 15 seconds, and elongation at 72 °C for five seconds, ending with one cycle for the final elongation at 72 °C for 10 minutes.

Table 1 Microsatellite loci that were used in this study, as described by literature

Marker	Tm	Sequence	Dyes	Alleles	Repeat sequence	References
CAU3	58.5	F: AACTAAGTATAGCCCTGTTACA R: TGCGAGTCTTTCTAGTTCTAC	VIC	6	$(CA)_9$	Tang *et al.*, 2003
CAU14	58.5	F: ATTTAACTTCTCTAAGGCACTC R: GAGGAGCAATTCAGACAGAC	6-FAM	14	$(CA)_{16}$	
CAU17	58.5	F: CGTAAACCCAGATAATCACAA R: AGTGGCATTGTAGCTCTTCA	NED	11	$(CA)_{22}$	
CAU42	61.5	F: AGTCCAGCCCGCATACAC R: CCTCTGTGGAGAGAACTGTGTG	PET	7	(CA)10	
CAU83	68.5	F: AAACAAGCCGCTAGTGAGGA R: TGCAGACTCAGACCAGCATC	PET	8	$(AC)_{16}$	
CAU85	60.5	F: GAGGTGCCTGTCTTGTTTAC R: AAAAGCACCTTCCCACATTG	NED	16	$(AC)_{26}$	
CAU128	64.9	F: TAAACACAAACAGACACAGAC R: TAACTTTGTGGCAACCAGTAG	6-FAM	4	$(AC)_{11}$	Huang *et al.*, 2008
CAU129	67.9	F: GGCACAATTTCCTACCAAGC R: GGGACTGATGCTGTCTGGTT	PET	11	$(AC)_{22}$	
CAU131	64.9	F: CCAATTCCGTGCATATGTGT R: TGTCAGGTGTTTCTGCATCA	VIC	10	$(CA)_{20}$	
CAU133	60.7	F:GGAAGATCCTTGCTGTTGGT R: TGGACTGTTATCTGGCGATG	6-FAM	7	(CA)15	
CAU144	60.7	F: ATATGCATGTGAGTATAAACAC R: CTGGGGAGCAGAGTCACC	PET	10	$(AC)_{17}$	
LIST005	55	F: ATGGTGCTTTCCAGTGGTGTGC R:	6-FAM	10	$(TG)_2CG(TG)_{10}$	Kumari & Kemp, 1998
LIST009	55	F: CATTGCAAACACTCTGCTGC R: TGAACGACAGGGTTATTGGC	6-FAM	13	$(CA)_{14}CG(CA)_3CG(CA)_3$	
LIST0011	58	F: ACTGAAGTTTCCTTCTCCCC R: TTCCTGAAGCAACCACAC	PET	10	(GT)24	
OSM1	57	F: AATCTGCCTGCAAAGACCAG R: TCCCAGTCTTGAAGTCAGCA	6-FAM	9	$(CA)_{17}$	Kimwele *et al.*, 1998
OSM2	57	F: AAGCCACGGCAATGAATAAG R: CCTCAACCATTCTGTGATTCTG	NED	6	$(CA)_{22}$	
OSM3	57	F: ATCTCCTTTGCTGGTGCAAT R: CCGGGGGGATTTCTTATGT	VIC	4	$(CA)_{15}$	
OSM4	56	F: ATCACTTTGCTGAAGTCAAAGG R: CTAACAGAGATCTGGGCGGA	PET	5	$(CA)_{16}$	
OSM5	59	F: GTGGATCAGTTCAATCCTTGC R: GCCCAAGAAAATGATGGAGA	NED	6	$(CA)_{20}$	
OSM7	58	F: AGCATACACATGCAGACCCC R: TGTTTCCTGCCATTCTGTCA	VIC	7	$(CA)_{16}CT(CA)_5CT(CA)_{25}$	
VIAS-OS4	53.7	F: CTCCTGGATGTTCTAGCAGT R: CTCCTTGTCCAGCCATATAC	VIC	12	$(GTGTAT)_2(GT)_9$	Kawka *et al.*, 2007
VIAS-0S14	49.9	F: CACTTCTCCGAATTTTAAAAGG R: AGGAAGAGATGTGGAGTCCC	6-FAM	18	$(AC)_{21}$	
VIAS-OS29	55.1	F: TTTTCGTCTTCCACCCACTG R: CTGCTTCTTCCGTGTGTGTC	PET	18	$(AC)_{13}GG(AC)_6GG(AC)_4$	

The DNA was amplified by targeting 23 microsatellite loci that had already been reported in literature. The microsatellite primer sequences were selected out of the studies by Kimwele *et al.* (1998), Tang *et al.* (2003), Kawka *et al.* (2007) and Huang *et al.* (2008) and are described in Table 1. Microsatellite loci with a high observed heterozygosity, >4 alleles and di-nucleotide repeats were selected for this analysis. The

Fluorescent PCR products were electrophoresed using the 3130xl Genetic Analyzer (Applied Biosystems®) available at the Central DNA Sequencing Facility at Stellenbosch University. The alleles were scored using ABI Prism® Genemapper software Version 4.0 (Applied Biosystems®).

Amplification was successful for 21 of the 23 microsatellite loci that were tested initially. *CAU42* and *LIST0011* failed to amplify and were therefore excluded from all further analysis. The *CAU133* and *VIAS-0S14* microsatellite loci were also excluded from the analysis because they were monomorphic in all the samples that were analysed. The remaining 19 microsatellite loci were therefore used in this study. Information on these loci is listed in Table 2. Three individuals, two of the SAB breed and one of the ZB breed, were also omitted from the data, as many of their loci failed to amplify. This might be owing to poor DNA quality or insufficient primer binding conditions during the PCR reaction.

The 19 microsatellite loci were checked for null alleles or scoring errors by the software program Microchecker version 2.2.3 (Van Oosterhout *et al.*, 2004). After this check, the microsatellite loci were evaluated for possible signs of selection using the F_{ST}-outlier method of Beaumont & Nichols (1996) implemented in Lositan Version 1 (Antao *et al.*, 2008). Genetix version 4.0.5.2 (Belkhir *et al.*, 2004) was then used to test for the number of alleles as well as the expected and observed heterozygosity of each microsatellite locus. An analysis was performed without four loci that were subjected to positive selection and also excluding the two loci containing null alleles.

The genotypic and allelic frequencies were estimated using the software program GDA Version 1.1 (Weir, 1996). The microsatellite toolkit analysis (Park, 2001) was used to determine the average number of alleles, expected and observed heterozygosity and fixation index with the respective standard deviations for each subpopulation. The fixation index (F_i) is a measure of excess homozygosity within a population and is interrelated with the inbreeding coefficient (Hamilton, 2009). Genetic differentiation was measured in terms of pairwise F_{ST} values calculated in Genetix Version 4.0.5.2 (Belkhir *et al.*, 2004). The Nei's statistic (Nei, 1972) was calculated using GENEPOP version 4.0 software (Raymond & Rousset, 1995).

Results and Discussion

Microsatellite loci, CAU144 and LIST005 were found to possibly contain null alleles, and four loci (VIAS-0S14, CAU3, OSM4 and LIST009) were possibly under positive selection at the 95% confidence level. The exclusion of the latter four loci from the analysis did not affect the results, that is, whether there are genetic differences between the breeds. The findings presented here thus report on the overall findings, that is, irrespective of whether 15 or 19 microsatellite loci were used. The results for the microsatellite marker polymorphisms are shown in Table 2. All 19 loci used were polymorphic. A high number of alleles (28) were observed for *CAU85* in contrast to the number of alleles (16) that were reported previously (Tang *et al.*, 2003). Microsatellite locus *LIST009* had 27 alleles in comparison with the 13 alleles previously reported by Kumari and Kemp (1998). The number of alleles observed for *OSM7* was 24 vs. the seven alleles previously reported by Kimwele *et al.* (1998). These differences in number of alleles may be owing to allele drop-out, which was not reported in the above studies, because of fewer PCR cycles during their annealing stage or because of possible mutations occurring at these loci (Beuzen *et al.*, 2000).

Genetic diversity can be described by the mean number of alleles per locus as well as by the mean expected and observed heterozygosity of those alleles. A total of 263 alleles were observed across the three ostrich breeds. The average number of alleles per locus was 13.8 (Table 2). The expected heterozygosity amounted to 0.81 and the observed heterozygosity 0.69 for all the loci across all three breeds. This implies that there is a substantial amount of genetic diversity within the three ostrich breeds farmed with commercially in South Africa.

The average numbers of alleles per population were 8.8 for the SAB, 9.4 for the ZB and 6.2 for the KR, which gives an average of 8.1 alleles across all three breeds, with a mean expected heterozygosity of 0.74 and an observed heterozygosity of 0.69 (Table 3). Observed heterozygosity was the highest in the SAB (0.72 ± 0.019) breed, whereas it was the lowest for the ZB (0.68 ± 0.019) and KR (0.69 ± 0.026) breeds. These results are consistent with those of Kawka *et al.* (2007), who also found that the SAB breed had the highest level of heterozygosity and the KR the lowest. This may be because the SAB is a composite breed derived from northern African *(S. c. camelus)* and southern African *(S. c. australis)* ostriches (Deurden, 1913). The SAB genetic resource located at Oudtshoorn Research Farm also originated from different genetic resources (Bunter, 2002), possibly contributing to a higher diversity pool for this breed. The lower genetic variability observed in the ZB population may be because no prior knowledge was available of

possible kinship relationships between individuals within the ZB breed. These birds could therefore be related to one another. The fixation index reported in this study is 0.05, 0.13 and 0.01 respectively for the SAB, ZB and KR breeds (Table 3). The estimate for fixation index within the ZB breed is the highest (F_i = 0.13), which are indicative of a closer degree of relatedness between individuals within this breed. The KR has the smallest F_i measure of 0.01, indicating limited inbreeding within this breed.

Significant differences were observed in terms of the genetic resemblance among the three breeds (Table 4). The SAB and ZB were genetically more similar to each other (F_{ST} = 0.10 and Nei = 0.49, P <0.05). The largest genetic distance were estimated between the ZB and KR breeds (F_{ST} = 0.13 and Nei = 0.61, P <0.05). This result was unexpected, because the areas of origin of the ZB and KR breeds are geographically closer than those of the SAB and KR breeds. Nevertheless, the largest genetic distance in the literature was previously reported between the SAB and KR breeds and the smallest between the ZB and KR breeds (Kawka *et al.*, 2007). The inconsistency of results might be because a bigger population size and a wider variety of microsatellites that were used in this study when compared with that of Kawka *et al.* (2007). An F_{ST} value of zero suggests that the variance of the allele frequencies within each population is similar (Hartl & Clark, 1997; Holsinger & Weir, 2009). All these F-statistic values fall in the range of 0.05 to 0.15 which is interpreted as moderate genetic differentiation between the breeds (Hartl & Clark, 1997) and therefore the variance of the allele frequencies among the SAB, ZB and KR differs (P <0.05). The genetic differentiation of the three breeds is illustrated in Figure 1.

Table 2 Observed results for microsatellite loci across the South African Black, Zimbabwean Blue and Kenyan Redneck ostrich breeds

Marker	Alleles	Observed Size	Expected size	Expected heterozygosity	Observed heterozygosity	Fixation index
CAU3	5	120	125	0.67	0.63	0.07
CAU14	10	155	178	0.85	0.83	0.03
CAU17	12	197	180	0.86	0.72	0.20
CAU83	8	217	218	0.76	0.58	0.30
CAU85	28	287	276	0.95	0.92	0.03
CAU128	8	223	231	0.61	0.61	0.01
CAU131	12	120	125	0.81	0.72	0.11
CAU133	4	196	201	0.71	0.71	0.01
CAU144	9	166	167	0.74	0.45	0.40
LIST005	16	224	197	0.88	0.63	0.30
LIST009	27	328	199	0.94	0.80	0.20
OSM1	16	141	110	0.84	0.80	0.06
OSM2	17	187	121	0.90	0.66	0.30
OSM3	7	152	232	0.59	0.58	0.03
OSM4	13	159	134	0.83	0.55	0.3
OSM7	24	241	215	0.93	0.89	0.04
VIAS-0S4	14	272	268	0.80	0.53	0.3
VIAS-OS14	16	243	245	0.88	0.74	0.2
VIAS-0S29	17	155	173	0.89	0.88	0.01
Average	13.8 ± 1.6			0.81 ± 0.2	0.69 ± 0.03	0.15 ± 0.03
Total	263					

Table 3 Mean (± SD) number of alleles, expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (F_i) for the South African Black (SAB), Zimbabwean Blue (ZB) and Kenyan Redneck (KR) breeds

Population	Alleles	Expected heterozygosity	Observed heterozygosity	Fixation index
SAB	8.8 ± 4.06	0.75 ± 0.030	0.72 ± 0.019	0.05
ZB	9.4 ± 3.96	0.78 ± 0.034	0.68 ± 0.019	0.13
KR	6.2 ± 2.29	0.69 ± 0.024	0.69 ± 0.026	0.01
Average	8.1	0.74	0.69	0.07

Table 4 Mean Nei's standard genetic distances, pairwise F-statistic value as a measure of genetic variation between the South African Black (SAB), Zimbabwean Blue (ZB) and Kenyan Redneck (KR) ostrich breeds

Breeds	Nei's genetic distance	Pairwise F-statistic
SAB and ZB	0.49 (P <0.05)	0.10 (P <0.05)
SAB and KR	0.51 (P <0.05)	0.12 (P <0.05)
ZB and KR	0.61 (P <0.05)	0.13 (P <0.05)

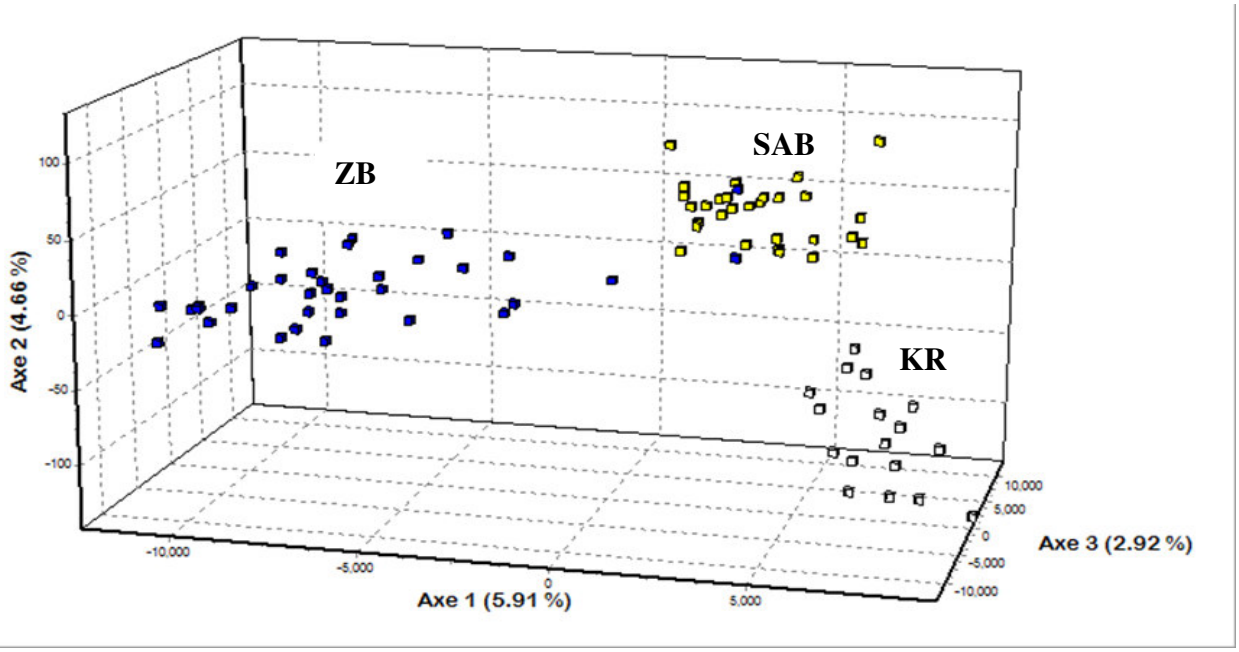

Figure 1 A three-dimensional graph of a factorial correspondence analysis (FCA) to illustrate the genetic differentiation among the SAB, ZB and KR breeds.

Conclusion

The study indicates considerable genetic differentiation among the three ostrich breeds. The SAB breed seems to have the highest level of genetic variation. This high genetic variation provides capacity for genetic improvement within that breed. Genetic improvement has been achieved within the SAB breed in a line that was selected for chick production, which resulted in an increase of 3.1% per annum (Cloete *et al.*, 2008a). The SAB founder population has also been sourced from different origins and initially different lines ("commercial" vs. feather) to form the basis of the population represented in this study. The ZB and KR

exhibited lower levels of genetic variation. In the case of the ZB breed, this result may stem from the fact that the resource population did not have any ancestral information albeit sourced from separate commercial entities. The low levels of genetic variation within the ZB and KR breeds can be seen as a benefit, as crossing of separate populations with a higher level homozygosity is expected to lead to increased levels of heterosis. Genetic differences obtained among the SAB, ZB and KR serve as confirmation of the phenotypic differences reported among the breeds (Jarvis, 1998). Application of this knowledge can lead to economically viable commercial crossbreeding programmes based on scientific principles. Hybrid vigour stemming from crossbreeding can lead to improved reproduction of crossbred females, as well as an improved survival of crossbred chicks, as was observed by Engelbrecht *et al*. (2008). Since these improvements stem from the non-additive part of genetic variation, it is likely to be well adapted to commercial situations where terminal crossbreeding systems can be applied.

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