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# Molecular Genetics Unveiled Unknown Family Relationships and Hybrids in an Ex-Situ Colony of African Penguins (*Spheniscus demersus*)

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## Abstract

Genealogical relationships among colony members, inbreeding status, and presence of hybrids are crucial data that can assist zoo curators in captive colony management and decision-making on relocation for reproduction. This study employed molecular markers to study a large colony (*n* = 56) of African Penguin hosted in an Italian biopark. A panel of 15 STRs (single tandem repeats) was selected, and genotype data were analyzed using COLONY software to determine parentage relationships and compare the existing studbook information to a pedigree built from genetic analyses. The existence of extra-pair mating and the presence of hybrids were investigated: discrepancies in kinship relationships emerged following molecular parentage analysis and 10 unknown genetic relationships were revealed. Infidelity of one member of the pair was observed in 6 cases and extra-pair copulation was assessed by genetic analysis in 2 episodes. One member of the colony was found to be a hybrid (*S. demersus* × *S. humboldti*); his progeny, derived by extra-pair copulation, was traced. Three other hidden hybrids were discovered and assessed using the identified candidate private alleles. Overall, our results demonstrate that molecular methods to confirm parentage and analyze relatedness among colony members are a valuable tool to complement studbook-based genetic management of African penguin captive populations. Because a variety of behavioral dynamics (e.g., extra-pair mating) can make observations ineffective in some species and because molecular markers outperform studbook in identifying the presence of hybrids, reliance on studbook information alone is not recommended.

**Keywords:** COLONY software, extra-pair mating, hybrids, pedigree, STRs, Zoo

The African penguin (*Spheniscus demersus*) is a seabird classified as endangered by the International Union for Conservation of Nature (BirdLife International 2015) due to the rapid decrease in wild populations during the 20th century. Since this trend shows no signs of reversing, immediate conservation actions are needed to prevent further decline. Despite multiple conservation interventions,

the population counts 50 000 mature individuals (roughly about 75 000–80 000 adult individuals in total) spread along the coast of Namibia and the South Africa where the species breeds (BirdLife International 2015). The decline has been attributed to excessive egg and guano harvesting (Shelton et al. 1984), competition for food with seals and commercial fisheries (Frost et al. 1976; Crawford et al. 1992), oil spills (Morant et al. 1981; Adams 1994; Underhill et al. 1999), lack of prey species that influence breeding success (Crawford et al. 2006), loss of habitat, and climate change affecting prey distribution (Boersma 2008; Crawford et al. 2011).

African penguins breed well in captivity, and ex situ populations fulfill several different roles in conservation efforts, including public education, resources for scientific discovery (e.g., Favaro et al. 2014, 2015), and sources for supplementation or restoration of in situ populations (Lacy 2009). Regarding this last role, conservation of the genetic heritage of African penguins should be a priority for zoological gardens. The use of captive colonies for restoring wild populations has made it necessary to assess their genetic status, for which regional studbooks have been established as part of preservation programs throughout the world. A studbook is a database that collects the pedigree information and the major events of an individual's life history in a defined population (Earnhardt et al. 2005). Studbooks provide data for pedigree analyses, which are the foundation for analyzing and managing the demographic and genetic health of captive populations (Ballou et al. 2010). Accurate pedigrees yield essential information on inbreeding, kinships among individuals, and the distribution of individual founder contributions to a population (Ivy and Lacy 2010).

Therefore, studbooks are the first tool used to genetically manage captive animals and the selection of breeding pairs is a key step in growing sustainable population that meet conservation goals. However, some confounders (i.e., unknown founders, extra-pair mating, or undisclosed hybrids) may prevent to maximize genetic diversity and minimize inbreeding. In such cases, pedigree analysis by molecular tools can help decision makers to detect missing and incorrect information, overall improving breeding programs (Ivy and Lacy 2010; Ferrie et al. 2013).

In Europe, the management of captive animals, just as for other endangered species, is coordinated by the European Endangered Species Programme (EEP) with the support of the European Association of Zoos and Aquaria (EAZA). Assisted by a group of experts, the EEP coordinator decides on the management of mating pairs, selecting which couples should be allowed to mate and which individuals should be moved to another park to create new mating pairs. This kind of management has several critical points: existing populations have a limited number of individuals and originated from a few founders (Fienieg and Galbusera 2013). By convention, the curator of a collection revises the animal pedigrees when a new individual is born. This is easy for African penguin colonies due to their monogamy. Indeed, between 80% and 90% of penguins in the wild retain the same mate at each mating season (Randall 1983). However, extra-pair mating can also occur, as pointed out by the Penguin (*Spheniscidae*) Care Manual (Schneider et al. 2014) published by the Association of Zoos and Aquariums. The Manual calls attention to the fact that several penguin species have sometimes been hosted in the same enclosures. This unwise practice may have led to the mating (also extra-pair mating) between 2 species and to the spread of hybrid subjects. Another problem in colony management is erroneous sex assignment of colony members by zoo keepers. Penguins are not sexually dimorphic. Generally, the male is larger than the female. Since comparison can be made only when the animals pair, sex assignment in juveniles is problematic.

Molecular methods could help to reduce uncertainty since they can be effectively employed to: 1) identify unknown parentage relationships; 2) assess the presence of hybrids; 3) assign sex to the components of the colony; and 4) verify the accuracy of the existing pedigrees. In the present study, we employed molecular markers to study a large colony of African Penguin hosted in an Italian biopark. Genetic variability and the inbreeding coefficient were evaluated, and parentage relationships were determined to compare the existing studbook information with a pedigree built from genetic analyses (GP). Furthermore, molecular sexing of each individual was carried out and the existence of extra-pair mating in the colony was investigated. Finally, mtDNA analysis of a penguin showing Humboldt penguin phenotypical characters was carried out to check whether it could be a hybrid. His progeny, derived by extra-pair copulation, was traced, and the "Humboldt's candidate private alleles" were followed to look for other "hidden" hybrids.

Our findings provide new baseline knowledge about ethology and mating behavior of African penguins that have important implications for the correct management and breeding of these seabirds in captivity, and provide a valuable contribution to studies in other captive populations. Especially in group-living species, in which pedigree analysis for the genetic management is far from being trivial, there is a need to develop methods and practical computing tools to analyze pedigrees in order to maintain genetic diversity of the ex situ population, main goal of an ex situ breeding program (Jiménez-Mena et al. 2016). Moreover, our approach allowed us to highlight aspects of species biology (i.e., extra-pair fertilizations, polygamy, cuckoldry) important for natural populations and to disclose the potential problem of hybrids particularly important if the ex situ population is considered a source of integration or restoration of populations in situ.

## Materials and Methods

### Penguins and Housing

The studied colony consisted of 3 groups of African penguins (56 birds in total) from 3 different zoological gardens: Wilhelma Zoo (WZ) (Germany), Bird Park Avifauna (BPA) (the Netherlands), and Artis Royal Zoo (ARZ) (the Netherlands). The animals had been grouped in South Lake Wild Animal Park (SLWAP) (UK) and then transferred to the Zoom biopark in Cumiana (Piedmont, northwest Italy). Ten penguins from WZ, 17 from BPA, and 8 from ARZ arrived in SLWAP between 2004 and 2005.

### Genetic Analysis

**DNA Extraction and Molecular Sexing**
DNA was extracted from EDTA-treated whole blood collected from 56 penguins by means of the NucleoSpin® Blood kit (Macherey-Nagel). DNA was quantified with a nanophotometer (Vivaspec LS, Sartorius) and then stored at −80 °C. All samples were submitted to molecular sexing, a modified version of the protocol described by Griffiths et al. (1998) was used: the P2 primer was labeled at the 5′ end with a fluorophore (HEX) to detect the difference in length of the chromobox-helicase-DNA-binding gene (*CHD*) by capillary electrophoresis. The PCR reaction was carried out in a total volume of 25 μL containing 50–60 ng of DNA template, 1.5 mM $MgCl_2^+$ buffer, dNTPs (0.2 mM each), 0.3 μM of each primer, and 1.5 UI of HotStarTaq® DNA polymerase (Qiagen). The PCR program was: 95 °C for 15 min;

40 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, extension at 65 °C for 1 min, and a final extension step at 65 °C for 5 min. Amplicons were diluted 1:1000 with ultrapure water and then analyzed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems™). Fragment sizing was carried out using the ROX™ 500 size standard (Applied Biosystems™) and Genemapper™ v. 3.7 software (Applied Biosystems™).

### Microsatellite Genotyping

All samples (*n* = 56) were submitted to parentage analysis: 26 microsatellites were initially evaluated starting from a panel of those characterized in *S. demersus* or in other penguin species and reported to be polymorphic (PNN01, PNN03, PNN05, PNN06, PNN07, PNN08, PNN09, PNN12, Sh1Ca16, Sh1Ca17, Sh2ca22, Sh2Ca21, B3-2, H2-6, G2-2, G3-6, M1-11, EMM1, EMM2, EMM3, EMM5, Em1, Em2, Em6.2, Em8, Em12.2). Supplementary Table 1S presents the characteristics of the microsatellite loci.

Simplex PCRs were initially carried out to assess positive amplification of each locus. For the evaluation of markers studied in species different from *S. demersus*, only those shown to be polymorphic (i.e., amplicons of variable length in several subjects) were selected. PCR products were then sequenced to confirm the presence of STRs. The forward primer of each selected marker was dye labeled at the 5′ end; different fluorophores (FAM, HEX, and ATTO550; Eurofins Genomics) were used for multiplex PCR before capillary electrophoresis.

Genotyping was carried out using 15 selected polymorphic microsatellite loci by performing 3 different multiplex reactions: multiplex 1 (PNN01, PNN09, PNN12, B3-2, Sh1Ca16, and Sh1Ca17), multiplex 2 (PNN05, PNN06, PNN07, PNN08, and Sh2Ca22), multiplex 3 (Em 1, Em 6.2, Em 8, Em 12.2).

Homozygous samples were amplified 3 times to limit the large allele drop-out effect. PCR amplifications were carried out using the HotStarTaq® DNA polymerase Kit (Qiagen) in a total volume of 10 µL. The reaction mix of multiplex 1 was prepared as follows: 100 ng of template, 1X PCR buffer, 1X Q-solution, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 UI of Taq polymerase, and 0.3 µM of each primer. The reaction mix of multiplex 2 was composed of: 100 ng of template, 1X PCR buffer, 1X Q-solution, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 UI Taq polymerase, 0.3 µM of each primer for markers PNN05, PNN06, and Sh2Ca22, 0.2 µM of each primer for marker PNN08, and 0.45 µM of each primer for marker PNN07. The reaction mix of multiplex 3 was prepared using: 100 ng of template, 1X PCR buffer, 1X Q-solution, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 UI of Taq polymerase, and 0.45 µM of each primer. A reaction without any DNA was added as negative control to each run to assess absence of contamination. PCR cycles started with a denaturation step at 94 °C for 15 min, followed by 45 cycles at 94 °C for 1 min, at 55 °C (multiplex 1) or 53 °C (multiplex 2) or 60 °C (multiplex 3) for 40 s, at 72 °C for 40 s, and a final extension step at 72 °C for 30 min. PCR products were analyzed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems™). Fragment sizing was carried out using ROX™ 500 size standard (Applied Biosystems™) and Genemapper™ v. 5 software (Applied Biosystems™).

### Mitochondrial DNA Sequencing

Specimens from 15 out of 56 penguins were sequenced to identify their species. Species identification was carried out by direct sequencing of a 421 bp portion of the mitochondrial cytochrome b (*cytb*) gene (Verma et al. 2003). PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 (ThermoFisher) and analyzed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems™). The sequences were compared with those available in GenBank by means of a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and species was assigned on the basis of a similarity ≥99%.

### Statistical Analysis

FSTAT v. 2.9.3.2 (Goudet et al. 2001) software was used to test for evidence of linkage disequilibrium between pairs of loci and to calculate the number of alleles, the allelic richness at each locus, and the inbreeding coefficient (F*is*) value for each locus and the entire population. The *P* value was calculated by setting 1000 randomizations per locus.

CERVUS software v. 3.0.7 (Kalinowski et al. 2007) was employed to calculate the summary statistics including: observed heterozygosity (Ho); expected heterozygosity (He); polymorphic information content (PIC); the average probability that the set of loci would not exclude an unrelated candidate parent from parentage of an arbitrary offspring when the genotype of the other parent is unknown (NE-1P) or when the genotype of the other parent is known (NE-2P); the average probability that the set of loci would not exclude a pair of unrelated candidate parents from parentage of an arbitrary offspring (NE-PP); the average probability that the set of loci would fail to differentiate between 2 unrelated individuals (NE-I); the average probability that the set of loci would fail to differentiate between 2 randomly-selected full siblings (NE-SI); and the estimated null allele frequency ($F_{(Null)}$).

To assess parentage among the colony individuals and verify the pedigree obtained by observations in captivity, genotype data were analyzed with COLONY v. 2.0.5.9, which implements full-pedigree maximum likelihood methods (FL) to simultaneously infer sibship and parentage among individuals based on multilocus genotype data; candidate parents are assigned at 95% confidence (Wang 2004). All runs were carried out with the FL analysis method assuming polygamy as mating system without inbreeding. The high likelihood precision option and medium-length runs were set. Since the dataset was small and included families assumed to be very small (1 to 3 offspring per sibship), multiple runs with 3 different random number seeds were performed to check/confirm software output reliability, as suggested by Wang (2004). Studbook data were systematically used to exclude paternity and maternity on the basis of date of birth, date of death, and date of relocation (i.e., offspring had to have been born after parents but before the death of the putative parent and at the same institution). Molecular assigned sex was used, and paternity and maternity exclusion data were supplied, with 3 years considered as breeding age. Two runs were carried out assuming a value of 0.0001 for both allelic drop out and genotyping error rates. The mistyping rate per locus (accounting for typing errors and mutations) estimated by the software for the input genotype data was provided in the subsequent runs. A total of 10 runs were carried out. When reconstructed consistently among runs, sibship and parentage were considered reliably inferred and taken into account to confirm colony pedigree. Results for best configuration, full-sib, maternity, and paternity were evaluated. The probabilities of attribution were obtained as the mean value of the probabilities calculated by the software in different runs.

## Results

### Molecular Sexing

Molecular sexing was successful for all 56 animals tested. Following our protocol, PCR reaction on male samples produced a 364-bp

fragment of the *CHD* gene and showed a single peak after capillary electrophoresis; female samples were characterized by 2 *CHD* gene fragments of different length (364 bp and 380 bp) with 2 peaks in the electropherogram. The analysis allowed sex assignment to 30 sex-unknown individuals, including newborns and prepubertal animals. Sex assigned by keepers was confirmed in 25 other animals, which proved wrong in one case: the penguin named Zin, considered a male, was found to be female. In total, the colony was composed of 30 males and 26 females.

## Microsatellite Data

Of the 26 microsatellites tested for kinship, 7 (PNN03, Sh2Ca21, G2-2, G3-6, M1-11, and EMM1 EMM3) showed no positive amplification in our samples, whereas 4 (H2-6, Em2, EMM2 and EMM5) were monomorphic and excluded from further analyses.

Parentage and population parameters were obtained by evaluating the genotyping data from a panel of 15 microsatellites (B3-2; PNN01; PNN05; PNN06; PNN07; PNN08; PNN09; PNN12; Sh1Ca16; Sh2Ca22; Sh1Ca17; Em1; Em6.2; Em8; Em12.2). Only 1 marker (PNN07) showed the absence of amplification in 2 samples (Ice and Picchio).

Table 1 presents the statistical parameters for each locus and the values obtained across all loci in the entire population, the average nonexclusion probability for candidate parents (NE-1P; NE-2P, and NE-PP), and the average nonexclusion probability for identity (NE-I and NE-SI). Only 5 markers showed a PIC less than 0.5 (B3-2; PNN05; PNN07; Sh2Ca22; Em6.2). There was no evidence of linkage disequilibrium between loci. Neither F*is* values calculated for each marker nor that of the whole population showed a statistically significant *P* value (*P* < 0.05). Frequency of the null allele ranged from −0.075 to 0.085 and was considered irrelevant.

Tables 2 and 3 report the results of best configuration, full-sib dyads, maternity, and paternity relationship, as described by COLONY software. Kinships reconstructed consistently among runs

were considered reliably inferred if assigned a probability higher than 95% (strict confidence). Relationships ascribed consistently among runs were considered confirmed with a relaxed confidence if assigned a probability between 80% and 95% and confirmed with low confidence if assigned a probability less than 80%. Finally, parentages not consistent among runs were considered unconfirmed.

The kinship assignments obtained after FL analysis were used to check the family tree described in the studbook. Analyses carried out using COLONY allowed us to confirm 74 kinships out of a total of 83 relationships identified through field observations or documentation. Two pairs of full-sib relationships reported by birth certificates and 7 family relationships derived from field observations were not confirmed. Among these, 2 fathers and 1 mother were assigned differently from the studbook reports. Interestingly, 10 unknown family relationships (not reported in the studbook) were detected by genetic data analysis.

Out of 26 full-sib pairs, 14 were confirmed with strict confidence, 2 with relaxed confidence, 8 with low confidence, and 2 full-sibs pairs were not confirmed (Tables 2 and 3, and Supplementary Figure 1S). FL analysis revealed that 2 couples of penguins (Alfa and Spirit; Irene and Rashida; hereafter, the penguin names will be used as sample IDs) were neither full-sib nor half-sib. Lastly, FL analysis indicated the presence of 5 additional full-sibs not reported in the studbook.

Out of 29 maternity kinships reported in the studbook, 17 were confirmed by the software with a probability >95%, 5 with relaxed confidence, and 7 with low confidence. Best configuration analysis and comparison of the genotypes supported maternity for all of these relationships. One mother (Amadi) was assigned differently from the studbook. Finally, analysis of the dataset showed the presence of one additional unknown maternity parentage (Rashida and Sky) supported by high probability.

Out of the 28 paternity relationships reported in the studbook, 12 were confirmed with a probability >95%, 4 with relaxed confidence, and 5 with low confidence. Seven paternity assignments

**Table 1.** Loci summary statistics

| Locus | Allelic Richness | He | Ho | Fis | Fis *P* value | PIC | NE-1P | NE-2P | NE-PP | NE-I | NE-SI | F(Null) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B3-2 | 3.999 | 0.230 | 0.232 | −0.011 | 0.605 | 0.217 | 0.974 | 0.880 | 0.785 | 0.607 | 0.788 | 0.005 |
| PNN01 | 4.000 | 0.576 | 0.589 | −0.023 | 0.660 | 0.524 | 0.826 | 0.665 | 0.493 | 0.231 | 0.522 | −0.007 |
| PNN05 | 2.000 | 0.086 | 0.089 | −0.038 | 1 | 0.082 | 0.996 | 0.959 | 0.923 | 0.840 | 0.917 | −0.014 |
| PNN06 | 4.000 | 0.680 | 0.786 | −0.157 | 0.982 | 0.615 | 0.752 | 0.590 | 0.420 | 0.165 | 0.454 | −0.075 |
| PNN07 | 2.000 | 0.199 | 0.222 | −0.116 | 1 | 0.178 | 0.980 | 0.911 | 0.845 | 0.663 | 0.817 | −0.053 |
| PNN08 | 5.000 | 0.682 | 0.732 | −0.075 | 0.855 | 0.625 | 0.738 | 0.570 | 0.387 | 0.156 | 0.451 | −0.046 |
| PNN09 | 4.000 | 0.742 | 0.643 | 0.135 | 0.063 | 0.686 | 0.688 | 0.515 | 0.342 | 0.119 | 0.412 | 0.067 |
| PNN12 | 5.000 | 0.623 | 0.643 | −0.032 | 0.711 | 0.580 | 0.781 | 0.605 | 0.413 | 0.184 | 0.487 | −0.018 |
| Sh2Ca16 | 4.000 | 0.629 | 0.607 | 0.035 | 0.405 | 0.565 | 0.790 | 0.633 | 0.461 | 0.200 | 0.488 | 0.020 |
| Sh2Ca17 | 7.963 | 0.629 | 0.589 | 0.063 | 0.256 | 0.589 | 0.769 | 0.590 | 0.390 | 0.176 | 0.482 | 0.036 |
| Sh2Ca22 | 4.000 | 0.578 | 0.571 | 0.012 | 0.516 | 0.481 | 0.832 | 0.717 | 0.578 | 0.274 | 0.532 | 0.003 |
| Em1 | 7.000 | 0.770 | 0.768 | 0.002 | 0.540 | 0.736 | 0.612 | 0.429 | 0.232 | 0.083 | 0.389 | −0.012 |
| Em6.2 | 3.000 | 0.257 | 0.25 | 0.027 | 0.501 | 0.238 | 0.968 | 0.870 | 0.771 | 0.572 | 0.766 | −0.005 |
| Em8 | 4.000 | 0.642 | 0.607 | 0.055 | 0.324 | 0.566 | 0.789 | 0.641 | 0.483 | 0.202 | 0.482 | 0.027 |
| Em12.2 | 5.000 | 0.602 | 0.5 | 0.170 | 0.051 | 0.562 | 0.796 | 0.620 | 0.427 | 0.197 | 0.501 | 0.085 |
| All/mean | | 0.528 | 0.522 | 0.012 | 0.313 | 0.483 | 0.044 | 0.002 | 0.00003 | 8.05 e−10 | 0.00011 | |

Table reports: allelic richness; observed (Ho) and expected (He) heterozygosity; PIC value; Fis (with its *P* value); average nonexclusion probability for 1 candidate parent (NE-1P); average nonexclusion probability for 1 candidate parent given the genotype of a known parent of the opposite sex (NE-2P); average nonexclusion probability for a candidate parent pair (NE-PP); average nonexclusion probability for identity of 2 unrelated individuals (NE-I); average nonexclusion probability for identity of 2 siblings (NE-SI); estimated null allele frequency (F(Null)). The mean values or the values calculated across loci on the entire population are also shown.

**Table 2.** Results of Best Configuration Analysis obtained using COLONY software

Best configuration

| OffspringID | FatherID | MotherID | OffspringID | FatherID | MotherID |
|---|---|---|---|---|---|
| ALFA | *1 | #1 | PICCHIO | *3 | #4 |
| AMADI | *2 | #2 | PISTORIUS | *3 | SORRISO |
| ASCARI | SOLDATO | LUPIN | PRINCIPE | *11 | #8 |
| BAAKO | HARLOCK | ISABIS | RAMA | PRINCIPE | VALENTINA |
| BABY | *3 | #3 | RASHIDA | *12 | #7 |
| BIANCANEVE | *4 | #4 | RAS | RENATO | RASHIDA |
| CHRIS | UMBO | BIANCANEVE | RED | RICO | ALFA |
| SOLU | SOLDATO | LUPIN | RENATO | *13 | #12 |
| ZOOMA | MALAKA | ZOE | RICO | RENATO | RASHIDA |
| GIGI | KOWANSKY | IRENE | KIR | KOWANSKY | IRENE |
| GUIZZO | MALAKA | ZOE | SKY | RENATO | RASHIDA |
| HARLOCK | *5 | #5 | SOLDATO | *14 | BABY |
| HARRIS | HARLOCK | ISABIS | SORRISO | *15 | #13 |
| ICE | PRINCIPE | VALENTINA | SPARROW | JENGO | ZURI |
| IRENE | *6 | #6 | SPIRIT | *16 | #14 |
| ISABIS | *7 | #7 | SPONGY | JUNIOR | RASHIDA |
| ISA | *2 | AMADI | TALE | *4 | #2 |
| JENGO | *6 | #8 | TED | HARLOCK | ISABIS |
| JOCKER | *8 | #9 | TOBA | KUSUBIRO | TALE |
| JUNIOR | UMBO | #10 | TOWECA | *1 | AMADI |
| KOWANSKY | *9 | #11 | TWIN | *17 | AMADI |
| KUBA | KUSUBIRO | BABY | UMBO | *18 | #5 |
| KURA | HARLOCK | ISABIS | VALENTINA | *19 | ALFA |
| KUSUBIRO | RENATO | RASHIDA | VICTOR | *18 | ZOE |
| LUPIN | *7 | #11 | VIOLET | RENATO | RASHIDA |
| MALAKA | *10 | #5 | ZIN | *15 | #13 |
| NANO | *2 | AMADI | ZOE | *20 | #15 |
| OCEANO | HARLOCK | ISABIS | ZURI | *11 | #8 |

When the inferred father/mother is not found among the candidates, the father/mother ID is given an index (starting from 1) prefixed with "*" ("#" for mother). Offspring sharing the same father ID (no matter whether the father is found in the candidate males or not) are paternal sibs, those sharing the same mother ID are maternal sibs.

recorded in the studbook were not confirmed; among these, 2 fathers were assigned differently (Umbo and Kusubiro). Analysis of individual genotypes revealed that failed paternity assignments were mainly due to the presence of mismatches or null alleles. Finally, the analysis revealed an unknown paternity: Sky is the son of Renato (and Rashida). All unknown relationships, although assigned with a probability between 80% and 90%, were confirmed by comparing the offspring's and parents' genotypes and by the best configuration obtained using maximum likelihood analysis.

### Mitochondrial DNA Sequencing

The *cytb* sequence obtained from Umbo's DNA showed a similarity of 99% with Humboldt penguin sequences deposited in GenBank, showing that Umbo belongs, at least for the maternal line, to the *S. humboldti* species. Similarities with *S. demersus* and *S. magellanicus* sequences were lower (98% and 97%, respectively). Moreover, we noted that Umbo's genotyping profile showed 8 alleles (in 7 markers) with a low frequency within the sampled population (Table 4). These alleles allowed Umbo's assignment to his progeny (2 sons and 1 grandson), even if their mtDNA was found to have a similarity of 99% with sequences of *S. demersus*. Three other subjects carrying more than one of these alleles were detected. Their mtDNA sequences (Malaka, Sorriso, and Zin) showed that they also belong to Humboldt penguin species. The penguins display no phenotypical characters of *S. humboldti* species and are probably

hybrids. The mtDNA sequences of 8 penguins having one or none of "Umbo's" alleles showed a similarity of 99% with *S. demersus* sequences deposited in GenBank.

### Discussion

We applied molecular genetics techniques to study a colony of African penguins hosted in an Italian biopark. Previous studies have investigated the use of molecular methods to assess parentage in captive colonies (Zucoloto et al. 2009; Ferrie et al. 2013; Edwards et al. 2014; Sakaoka et al. 2014). Broad use of molecular markers is reported for species identification and detection of parentage in wild animals (Koch et al. 2008; Aykanat et al. 2014) (for a review, see Fienieg and Galbusera 2013). We employed a systematic method of molecular analysis that allowed us to address conservation issues in captive animals: 1) sex determination; 2) description of genetic parameters of a colony; 3) parentage assessment; and 4) species and hybrid identification. Our study followed a circular "check and verify" method between molecular data and studbook information; this approach was successful thanks to the continuous "check and verify" dialogue between geneticists, biologists, and zoo keepers.

For example, our observation that the most uncertain parentage assignments (for probability and/or reproducibility of the data) were among the clusters of penguins from the 2 Dutch zoos prompted suspicion of untraced animal exchanges. We presented

**Table 3.** Results from Full Sib, Maternity, and Paternity assignment obtained using COLONY software

| Full-Sib reconstruction | | | Level of confidence | Maternity reconstruction | | | Level of confidence | Paternity reconstruction | | | Level of confidence |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BAAKO | KURA | 1.000 | strict | GIGI | IRENE | 1.000 | strict | KURA | HARLOCK | 1.000 | strict |
| BAAKO | TED | 1.000 | strict | KIR | IRENE | 1.000 | strict | RED | RICO | 1.000 | strict |
| GIGI | KIR | 1.000 | strict | KURA | ISABIS | 1.000 | strict | TED | HARLOCK | 1.000 | strict |
| KURA | OCEANO | 1.000 | strict | KUSUBIRO | RASHIDA | 1.000 | strict | GIGI | KOWANSKY | 0.993 | strict |
| OCEANO | TED | 1.000 | strict | KUBA | BABY | 0.999 | strict | KIR | KOWANSKY | 0.993 | strict |
| KURA | TED | 0.999 | strict | BAAKO | ISABIS | 0.999 | strict | SPARROW | JENGO | 0.986 | strict |
| KUSUBIRO | RICO | 0.999 | strict | OCEANO | ISABIS | 0.998 | strict | BAAKO | HARLOCK | 0.976 | strict |
| ZOOMA | GUIZZO | 0.999 | strict | GUIZZO | ZOE | 0.998 | strict | RAMA | PRINCIPE | 0.966 | strict |
| KUSUBIRO | RAS | 0.997 | strict | CHRIS | BIANCANEVE | 0.998 | strict | ICE | PRINCIPE | 0.957 | strict |
| KUSUBIRO | VIOLET | 0.997 | strict | RAS | RASHIDA | 0.997 | strict | KUSUBIRO | RENATO | 0.956 | strict |
| RAS | VIOLET | 0.993 | strict | VALENTINA | ALFA | 0.994 | strict | RICO | RENATO | 0.955 | strict |
| RICO | VIOLET | 0.983 | strict | RICO | RASHIDA | 0.985 | strict | RAS | RENATO | 0.955 | strict |
| HARRIS | OCEANO | 0.974 | strict | HARRIS | ISABIS | 0.981 | strict | KUBA | KUSUBIRO | 0.937 | relaxed |
| BAAKO | HARRIS | 0.953 | strict | TED | ISABIS | 0.969 | strict | VIOLET | RENATO | 0.924 | relaxed |
| HARRIS | KURA | 0.907 | relaxed | VIOLET | RASHIDA | 0.968 | strict | OCEANO | HARLOCK | 0.800 | relaxed |
| HARRIS | TED | 0.907 | relaxed | SPONGY | RASHIDA | 0.965 | strict | HARRIS | HARLOCK | 0.800 | relaxed |
| BAAKO | OCEANO | 0.75 | low | SPARROW | ZURI | 0.957 | strict | ASCARI | SOLDATO | 0.739 | low |
| RAS | RICO | 0.734 | low | VICTOR | ZOE | 0.907 | relaxed | JUNIOR | UMBO | 0.692 | low |
| AMADI | ISA | 0.676 | low | RED | ALFA | 0.846 | relaxed | ZOOMA | MALAKA | 0.564 | low |
| ICE | RAMA | 0.625 | low | TOBA | TALE | 0.832 | relaxed | SOLU | SOLDATO | 0.532 | low |
| ZOOMA | VICTOR | 0.573 | low | PISTORIUS | SORRISO | 0.800 | relaxed | GUIZZO | MALAKA | 0.470 | low |
| ASCARI | SOLU | 0.532 | low | ZOOMA | ZOE | 0.800 | relaxed | SPONGY | JUNIOR | 0.208 | low |
| GUIZZO | VICTOR | 0.435 | low | TWIN | AMADI | 0.771 | low | | | | |
| TOWECA | TWIN | 0.319 | low | TOWECA | AMADI | 0.763 | low | | | | |
| | | | | ICE | VALENTINA | 0.756 | low | | | | |
| | | | | NANO | AMADI | 0.721 | low | | | | |
| | | | | SOLU | LUPIN | 0.707 | low | | | | |
| | | | | ASCARI | LUPIN | 0.704 | low | | | | |
| | | | | RAMA | VALENTINA | 0.652 | low | | | | |

| New parentage assignments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| KUSUBIRO | SKY | 1.000 | strict | SKY | RASHIDA | 1.000 | strict | TOBA | KUSUBIRO | 0.850 | relaxed |
| SORRISO | ZIN | 1.000 | strict | ISA | AMADI | 0.959 | strict | SKY | RENATO | 0.874 | relaxed |
| SKY | VIOLET | 0.996 | strict | SOLDATO | BABY | 0.767 | low | CHRIS | UMBO | 0.819 | relaxed |
| RAS | SKY | 0.996 | strict | | | | | | | | |
| RICO | SKY | 0.836 | relaxed | | | | | | | | |

Only kinships reconstructed consistently among runs are reported. Results are ranked from the highest to the lowest probability of attribution. The level of assignment confidence, as described in the text, is reported. At the bottom of each section, the previously unknown attributions are also reported.

**Table 4.** Candidate private alleles of *S. humboldti*

| Marker | Allele size | Allele frequency |
|---|---|---|
| **B3-2** | 300 | 0.036 |
| **B3-2** | 306 | 0.076 |
| **PNN06** | 323 | 0.071 |
| **PNN08** | 143 | 0.045 |
| **Sh1Ca16** | 96 | 0.098 |
| **Sh1Ca22** | 91 | 0.036 |
| **EM1** | 227 | 0.080 |
| **EM6.2** | 239 | 0.089 |

Allele size of the candidate allele and their frequency in the studied colony are shown for each marker.

our results to the zoo curators; a more careful documental check confirmed the occurrence of animal exchanges between the 2 zoos before 2005. Although our work was based on known methods, the results obtained with the simultaneous use of both molecular genetics and whole studbook records underscore that zoos should systematically apply this approach to overcome the problem of missing genealogical data (especially in old collections). We are aware that the problems described here are not unusual to other conservation breeding programs. Furthermore, we demonstrate that the coordinated use of molecular and studbook data in penguins can allow for considerably more precise genetic management of species of conservation concern. Our kinship analysis, carried out for the first time on a colony of African penguins, allowed us to acquire relevant information not only for the colony itself but also for EEP and the management of this species in other European zoos. Our approach allowed us to highlight aspects of species biology that are hard to assess on wild colonies and provides a valuable contribution to studies in other captive populations and in such a context, we believe that our work should be considered in a broader perspective.

Descriptive parameters of genetic diversity demonstrated that the penguin colony is substantially in Hardy–Weinberg equilibrium. The $F_{is}$ value was not statistically significant. The negative value of the inbreeding coefficient and $F_{is}$ negative values for some single

markers could be explained by the crossbreeding of individuals from 3 original groups (Wilhelma and the 2 Dutch zoos), and by the presence of some hybrids carrying alleles probably characteristic of the different crossed species.

To compare the studbook to the GP, we built a pedigree based on the genetic parentage analyses provided by the COLONY software. We knew that not all the parents would have been sampled (some remained in the zoos of origin, other died), whereas a high number of full-sibs and half-sibs were supposed to be present in the colony. Since our aim was to verify the studbook records themselves, we decided not to use any assumption based on a priori knowledge of existing parentage relationships. Therefore, the COLONY software was selected since it uses sibship reconstruction and parental reconstruction approaches, allowing discrimination between the progenies deriving from single and from multiple mating. COLONY infers both sibship and parentage relationships among all sampled individuals jointly using a maximum likelihood method. Actually, previous studies proved that the FL method is the most accurate (Wang 2004, 2012; Wang and Santure 2009) and that COLONY outperforms other commonly used software in carrying out parentage analysis (Wang 2012; Ferrie et al. 2013; Harrison et al. 2013). The studbook was largely confirmed by the GP, although several discrepancies in kinship relationships emerged from the comparison of the 2 pedigrees. Using only molecular data (sex and genotype) and age information, 89% of the parentage relationships were reconstructed and 11% of the studbook data were not confirmed. One mother and 2 fathers were assigned differently from the studbook record. Molecular methods have the valuable ability to indisputably exclude relationships when mismatched genetic markers are found between offspring and presumed parents recorded in a studbook (Ferrie et al. 2013). For instance, 2 penguin couples (Alfa and Spirit, Rashida and Irene) were reported to be full-sib in the documentation accompanying the animals coming from ARZ. FL analysis of the genetic data showed no evidence of either full-sib or half-sib kinship, however. Furthermore, the GP showed 10 previously unknown family kinships. We hypothesized a documental error or an incorrect assignment to the same mating pair in the zoo of origin. In such circumstances, molecular methods could provide a useful tool for testing studbook records. Nonetheless, strict adherence to genetic data is not recommendable: besides scoring or sequencing errors, other limitations in the use of genetic data for parentage assignment can emerge when sampling of potential parents is incomplete or homozygosity reduces the informativeness of the markers. In our study, as frequently occurs in captive colonies, potential parents may not have been sampled and analyzed, because they were relocated or died. Moreover, when the number of founders is low, a high homozygosity in offspring could be observed and might explain the absence (or a low probability) of paternity assignment to the candidate father.

Genetic analysis allowed us to highlight aspects of species biology that are hard to assess by observations: extra-pair copulations, same sex couples, and hybrids *S. spheniscus* × *S. humboldti*. This information—unknown to the zoo curators—holds importance for the management of the population and for implementing knowledge about their ethology. During the 16-month observation period, 4 episodes of infidelity were recorded by field observations and parentage genetic analysis of the offspring confirmed extra-pair copulation in 3 cases. Two additional episodes of infidelity were revealed only by our genetic analyses. We detected the occurrence of extra-pair mating behavior in 5 individuals within a group of 35 broodstocks (14% prevalence). Although long believed a monogamous species, extra-pair copulation has been described in different penguin

species: the Humboldt penguin (*Spheniscus humboldti*; Schwartz et al. 1999) and the Adélie penguin (*Pygoscelis adeliae*; Hunter et al. 1995; Hunter and Davis 1998; Pilastro et al. 2001; Sakaoka et al. 2014). Of these studies, only that by Sakaoka and colleagues was conducted on captive animals. The kinship analysis we performed is the first to be carried out in a colony of African penguins housed in a European zoo (Valentina Isaja, member of the EEP African Penguin Committee; personal communication). In the wild, a study on the island of St. Croix, South Africa, showed that 92% of the African penguins kept the same partner in the next breeding season (García-Borboroglu and Boersma 2013).

Also, we discovered that the couple Zin and Biancaneve, which took care of the young Chris, actually consisted of 2 females. Both came from WZ, but we found no full-sib or half-sib parentage between them. Because of phenotypic appearance and behavior within the couple, Zin was believed to be male. According to genetic analysis, the young Chris turned out to be the son of Biancaneve and Umbo. Here, the molecular methods revealed a situation reported in other zoos worldwide (New York, Berlin, Toronto, Madrid, Ramat, among others) and in different penguin species, where couples of mates of the same sex live as a nesting couple and hatch eggs or care for adoptive offspring. In this case, one female of the couple paired with a male and then hatched the egg with another female.

Another interesting finding was that, based on field observations and studbook documentation, Amadi and Isa were considered full-sibs, though Amadi showed parental care towards Isa. Genetic data highlighted that Amadi is more likely Isa's mother (96% probability) rather than his sister (68% probability) and best configuration analysis confirmed this likelihood. Our study has contributed to a better understanding of the mating system in a zoo-living colony of African penguins. Unravelling the mating system of a species is essential for successful breeding, captive management, and conservation programs (Miño et al. 2009). Knowledge of the frequency of extra-pair paternity in colonially nesting species may have implications for the genetic management of captive avian populations (Ferrie et al. 2013). Moreover, we also identified some hybrids in the colony that were believed to be pure African penguins. The genus *Spheniscus* comprises 2 more extant species, other than Humboldt and African penguins: the Magellanic penguin (*S. magellanicus*) and the Galapagos penguin (*S. mendiculus*) (Ksepka and Thomas, 2012). Based on molecular and morphological dataset, Galapagos and Humboldt penguins are considered sister species, as are Magellanic and African penguins (Ksepka and Thomas 2012). However, among this genus, not being sister species does not seem to prevent hybridization: heterospecific paring and hybrids between Humblodt and Magellanic Penguins have been documented in the wild, where their habitats overlap along the cost of the southeastern Pacific Ocean (Simeone et al. 2009). Humboldt and African penguins do not share their habitat hence in the wild heterospecific pairing does not occur. In captivity, hybrids between Humboldt and Magellanic penguins, Humboldt and African penguins, and African and Magellanic penguins are reported (Thumser and Karron 1994; McCarthy 2006; Simeone et al. 2009). Concerns about the inclusion of more than one species of *Spheniscus* in mixed species zoo exhibits are expressed by the Association of Zoos and Aquariums in the last version of the Penguin (Spheniscidae) Care Manual (2014). In particular, Humboldt x African hybrids are considered quite common and recognized as partially fertile, since some individuals are known to have had offspring (McCarthy 2006). Awareness of the presence of hybrids is important for conservation purposes, especially as the

conservation of hybrids is highly controversial. Some studies and regulations argue that hybrids should not be conserved because they may threaten parental species, others assert that hybridization can be extremely beneficial for genetically depleted populations (Allendorf et al. 2001, 2004; Ellstrand et al. 2010, Kristensen et al. 2015 and references therein), The problem of hybrids is particularly important if the ex situ population is considered a source of integration or restoration of in situ populations, because they could cause a reduction of fitness (survival, growth, fecundity) in the endangered population and under this point of view, their presence needs to be mitigated avoiding mixed species exhibitions in zoos and using molecular methods to identify mixed and backcrossed individuals in old collections. Being present only in captivity, it is difficult to predict if the hybridization *S. demersus* × *S. humboldti* influences the fitness of the offspring. In zoos, environmental conditions are typically benign increasing the probability of survival for hybrids that might not otherwise survive in the wild. On the other hand, *S. demersus* × *S. humboldti* hybrids represent a case of anthropogenic hybridization involving species geographically isolated without reproductive isolation. In such a circumstance hybridization (as occurs in speciation reversal) could create novel, but advantageous, combinations of alleles that, in a changing world, allow mosaic hybrid species to occupy habitats and fill niches that their parent species could not (Kearns et al. 2018), moreover genomic selection strategies have been described to recover the genomic content of the original endangered population from admixtures (Amador et al. 2014) and *S. demersus* × *S. humboldti* hybrids would be beneficial to the parental species. In any case, human-mediated hybridization is increasing worldwide (Allendorf et al. 2001; Randi 2008) and hybrid management will need to be addressed in all conservation programs; given that with more generations of backcrossing, hybrids becomes genetically more similar to the parent species and may hold value for pure species conservation if they benefit the preservation of native diversity (Jackiw et al. 2015). For a more comprehensive review, we refer the reader to studies examining ethical and ecological implications of hybrid conservation (Lopez-Pujol et al. 2012; Jackiw et al. 2015) and cases of useful hybridization in species conservation (Fienieg and Galbusera 2013; Hamilton and Miller 2015). The point we wish to emphasize is that recognizing the presence of hybrids within collections is the first step in the decision-making process toward species conservation and mating management. For this purpose, molecular markers outperform studbook documentation, especially when private/diagnostic alleles are used to identify the crossed species. In the group studied here, some individuals showed African penguin phenotypical aspects but had both *S. humboldti* mitochondrial DNA and nuclear markers that could be private alleles of *S. humboldti*. Accordingly, they could be a first generation of hybrids or a backcross from a F1 with African penguin. Further analyses would be useful to assess the genetic divergence of these individuals from African penguin and Humboldt penguin.

In conclusion, our analysis of a colony of African penguins addresses the need to combine molecular data with observational and studbook information in the management of captive animal populations (Fienieg and Galbusera 2013). The family tree based on observation of pairs has been improved and modified thanks to genetic data analysis for this colony. Knowledge of the genetic background of the phenotype will help the colony curators to decide which animals to keep or move for breeding purposes, ultimately ensuring the genetic variability of captive penguin populations managed under ex situ conservation programs.

## Supplementary Material

Supplementary material can be found at https://academic.oup.com/jhered/.

## Data Availability

We have deposited the microsatellite genotypes underlying these analyses in Dryad (Dryad doi: 10.5061/dryad.9cv7kg7).

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Ethical Statement

The study was performed in compliance with the ethical guidelines for the management of research on zoos and aquariums animals (WAZA 2005), in accordance with Italian legislation and following the approval by the ethical committee of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (ref. no. 11168 of the 14th July 2014).

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