



Twins or not? Genetic analysis of putative twins in Antarctic fur seals, *Arctocephalus gazella*, on the South Shetland Islands

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ABSTRACT

Genetic analyses can reliably determine the relationships among putative cases of twins in pinniped species. These studies demonstrate that field observations of nursing twins may often be cases of adoption or foster nursing of unrelated pups. A recent study of Antarctic fur seals (*Arctocephalus gazella*) on South Georgia Island found that only 3 of 11 putative twin cases were truly twins. Here we report results of genetic testing of eight putative cases of twinning (twin siblings and mother) observed at Cape Shirreff (62°27'30"S, 60°47'17"W), Livingston Island, Antarctica. Parentage and relatedness analyses using 18 microsatellite markers confirmed six out of the eight cases as twins and two cases of adoption/foster nursing of unrelated pups. All twins analyzed were dizygotic and in five out of six cases, the twins were likely full siblings (relatedness coefficient, or $r_{xy\mu} = 0.46$, $\sigma^2 = 0.004$). In one case, the twins were likely half-siblings ($r_{xy} = 0.17$), supporting a previous finding of heteropaternality in Antarctic fur seals. This result suggests that mate infidelity during estrus may be common in Antarctic fur seals, which has implications for our understanding of this species's mating system. The twinning rate estimated at Cape Shirreff (0.12% or 6 twins per 4,965 births) is consistent with the scarcity of twin births observed in pinnipeds, which is associated with the high cost of nursing multiple pups in these animals.

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1. Introduction

Twins can be either monozygotic (MZ) or dizygotic (DZ), with MZ twins developing from one oocyte fertilized by a single sperm and DZ twins developing from two oocytes fertilized by two different sperms. In DZ twinning, the twins can share a single father or may have distinct fathers. In humans, single paternity is typical and as a result, DZ twins usually have the same genetic relationship as full siblings, sharing on average 50% of their genes (Hoekstra et al., 2008). However, the fertilization of two oocytes by sperm from different males, or DZ "heteropaternal superfecundation" (James, 1993) may also occur, which indicates partner infidelity during ovulation (Girela et al., 1997). In this case, DZ twins are fathered by distinct males and have the genetic relationship of half-siblings.

Twinning is considered rare among pinnipeds (Spotte, 1982). Nevertheless, twinning in phocids has been reported in elephant

seals, *Mirounga leonina* (Arnbom et al., 1997; Galimberti and Boitani, 1999; McMahon and Hindell, 2003) and Weddell seals, *Leptonychotes weddellii* (Gelatt et al., 2001). In otariids, twinning has been reported for several species: northern fur seals (*Callorhinus ursinus*), Cape fur seals (*Arctocephalus pusillus*), Antarctic fur seals (*Arctocephalus gazella*), California sea lions (*Zalophus californianus*), Steller sea lions (*Eumetopias jubatus*), southern sea lions (*Otaria byronia*) and New Zealand sea lions (Haase, 2007; Hoffman and Forcada, 2009; Maniscalco and Parker, 2009; Spotte, 1982). However, only a few pinniped twinning studies have confirmed relationships among mothers and pups using genetic analyses (Gelatt et al., 2001; Hoffman and Forcada, 2009).

Antarctic fur seal (*Arctocephalus gazella*) twinning has been recently examined by Hoffman and Forcada (2009). In their study at Bird Island, South Georgia, 11 putative cases of twins were analyzed and genetic twins were confirmed in only three cases. According to these authors, field observations of more than one suckling pup per mother must be foster nursing in the majority of cases, which can be fairly common at South Georgia (Hoffman and Amos, 2005a; Lunn, 1992). To further investigate this phenomenon, genetic analyses should be routinely undertaken to verify putative cases of twinning in pinnipeds (Gelatt et al., 2001; Hoffman and Forcada, 2009).

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Antarctic fur seal breeding populations are circumpolar, occurring at several islands south of the Antarctic Polar Front. Around the South American continent they are found at South Georgia, South Sandwich, South Orkney and the South Shetland Islands (Hofmeyr et al., 2006). As a consequence of over-hunting, Antarctic fur seals were extirpated from the South Shetlands by the end of the 19th century; the population has rapidly recovered to nearly 21,000 animals since the first birth documented at Cape Shirreff during the austral summer of 1958/59 (Hucke-Gaete et al., 2004; O’Gorman, 1961).

Antarctic fur seals have been intensively studied at Cape Shirreff for over a decade by researchers of the United States Antarctic Marine Living Resources (US-AMLR) Program. In recent years, putative twin cases have been observed and recorded in the field, providing an opportunity to investigate twinning in a recently recovered population of this species.

The objectives of this study were to: (1) investigate our ability to infer relationships among individuals via simulations of genotypic data, (2) genetically analyze putative twinning cases at Cape Shirreff, and (3) estimate twinning rates for the Cape Shirreff population and evaluate results within the context of Antarctic fur seal reproductive strategies.

2. Materials and methods

2.1. Sampling

The samples for this study were collected at Cape Shirreff (62°27' 30"S, 60°47'17"W) (Fig. 1), located south of the Drake Passage and on the northern coast of Livingston Island, the second largest of the South Shetland group. More specifically, Cape Shirreff is a low, ice-free peninsula of approximately 3.1 km² located between Barclay Bay and Hero Bay (Anonymous, 1994).

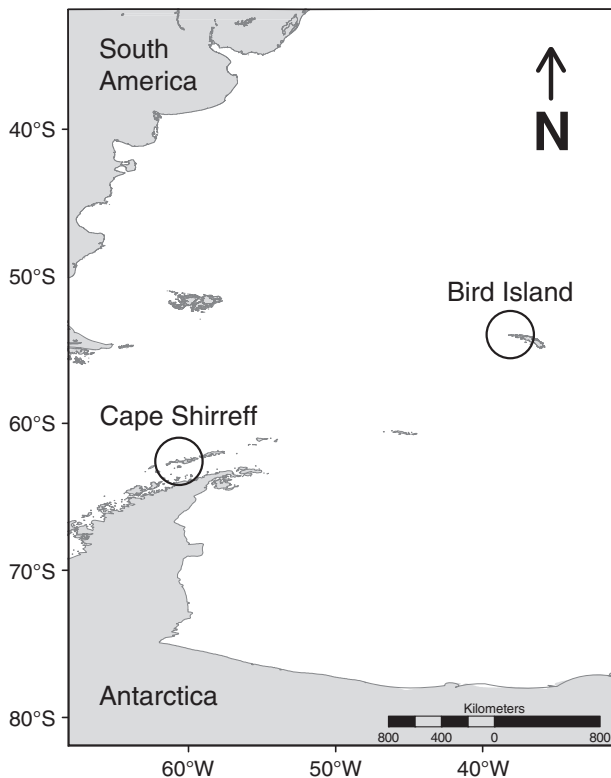


Fig. 1. The United States Antarctic Living Marine Research Program (US-AMLR) study site at Cape Shirreff, Livingston Island, Antarctica and the British Antarctic Survey Program study site at Bird Island, South Georgia.

All twinning cases were identified during the perinatal period (within one week of birth); they consisted of a pair of pups frequently observed nursing simultaneously on a single female. Candidate fathers were not sampled. Eight pairs of putative twins and their respective mothers (eight mothers and 16 pups, $n = 24$) were sampled during the austral summers of 2006–07; 2008–09; 2009–10. For a brief description of the putative twinning cases sampled in this study refer to Table 1. For the purposes of estimating allele frequencies within the Cape Shirreff Antarctic fur seal population, 94 pups ($n = 42$ males and 52 females) were sampled randomly during the austral summer of 2009–10.

Fur seal pups were sampled using 2 mm sterile biopsy punches, taking skin from the inter-digital membrane of the rear flippers. The biopsy punches were attached to a pole to collect tissue from 8 adult females (1 untagged, and 7 previously tagged; Dalton Jumbo Rototags, Dalton ID systems, UK). All tissue samples were stored in either 20% dymethylsulphoxide (DMSO) saturated with NaCl or 95% ETOH, and all procedures were conducted in compliance with Marine Mammal Protection Permit No. 774-1847-03 granted by the Office of Protected Resources, National Marine Fisheries Service, United States.

2.2. Laboratory procedures

Total genomic DNA was extracted from tissue samples using a NaCl precipitation method (adapted from Miller et al., 1988). After extraction, the genomic DNA was amplified for 18 microsatellite markers: Aa4, Hg3.7 (Gemmell et al., 1997); Ag10, Ag4, Ag7 (Hoffman et al., 2008), Agaz8, Agaz9 (Hoffman, 2009); Hl4, Lc28 (Davis et al., 2002); M2B (Hoelzel et al., 1999); Pvc29, Pvc78 (Coltman et al., 1996); ZcCgDh1.8, ZcCgDh4.7, ZcCgDh48, ZcCgDh5.8, ZcCgDh7tg, ZcCgDhB.14 (Hernandez-Velazquez et al., 2005). Amplification consisted of 15 μ l reactions containing: ~30 ng of template DNA, 2.0 μ M 1X ThermoPol reaction buffer (New England Biolabs, USA, catalog # B9013S), 1.5 μ M of dNTPs, 0.45 μ M of each primer (forward and reverse) and 0.5 u Taq DNA polymerase (New England Biolabs, USA, catalog # M0267L). The reactions were amplified in an ABI 2700

Table 1

Summary information on putative Antarctic fur seal (*Arctocephalus gazella*) twin groups collected in Cape Shirreff, Antarctica. Results of maternity and relatedness are also summarized.

Twin Group	LABID*	Seal description, age	Field season	Sex
CS1	62444	Adult Female (Tag# 392, 11 yo)	2006–07	F
	62445	Pup- putative twin (Mother 392)	2006–07	M
	62446	Pup- putative twin (Mother 392)	2006–07	M
CS2	62447	Adult Female (Tag # 395, 15 yo)	2006–07	F
	62448	Pup- putative twin (Mother 395)	2006–07	M
	62449	Pup- putative twin (Mother 395)	2006–07	F
CS3	62450	Adult Female (Tag# 391)	2006–07	F
	62451	Pup- putative twin (Mother 391)	2006–07	F
	62452	Pup- putative twin (Mother 391)	2006–07	F
CS4	62453	Adult Female (Tag# 412)	2006–07	F
	62454	Pup- putative twin (Mother 412)	2006–07	F
	62455	Pup- putative twin (Mother 412)	2006–07	M
CS5	78410	Adult Female (Untagged)	2008–09	F
	78408	Pup- putative twin	2008–09	M
	78409	Pup- putative twin	2008–09	M
CS6	78423	Adult Female (Tag# 440)	2008–09	F
	78405	Pup- putative twin (Mother 440)	2008–09	F
	78406	Pup- putative twin (Mother 440)	2008–09	M
CS7	92464	Adult Female (Tag# 448, 16 yo)	2009–10	F
	92546	Pup- putative twin (Mother 448)	2009–10	F
	92547	Pup- putative twin (Mother 448)	2009–10	M
CS8	92467	Adult Female (Tag# 450)	2009–10	F
	92550	Pup- putative twin (Mother 450)	2009–10	M
	92551	Pup- putative twin (Mother 450)	2009–10	M

*LABID corresponds to sample accession numbers for the Marine Mammal and Sea Turtle Molecular Research Collection, at the Southwest Fisheries Science Center, La Jolla, CA, USA.

thermocycler (Applied Biosystems, Foster City, California, USA) through an initial denaturing step of 97 °C for 3 min and 36 cycles of denaturing at 90 °C for 20 s, an annealing step at specific primer annealing temperatures (Tm; see Table 2 for specific primer Tms) for 30 s, and an extension at 72 °C for 20 s. Successful PCR reactions were processed following standard ABI protocols for fragment analysis. Samples were run on a 48-capillary, 3130xl ABI Genetic Analyzer, and resulting raw data files were analyzed and edited on ABI GeneMapper® v.4.0.

2.3. Data analysis

Microsatellite markers were assessed for the presence of null alleles using Microchecker v. 2.2.3 (Van Oosterhout et al., 2004). The dataset was also tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium (dememorization # = 100,000; 10,000 iterations per batch) using Genepop v. 4.0 (Raymond and Rousset, 1995). Marker scoring error rates were assessed by re-running 26% of the samples (samples were re-amplified and genotypes were re-scored): 12 samples from the 94 pups sampled for the population allelic frequency estimation and 24 samples corresponding to all putative twins and their mothers. The error rate was calculated as the number of mismatched calls divided by the total number of calls for the replicated samples per locus (as described by Bonin et al., 2004). Additionally, mismatched calls were triplicated to reduce error. An identity analysis was conducted on the 94 randomly collected samples used to estimate baseline allele frequencies within the population. This analysis was carried out to check for potential duplicate samples (animals mistakenly sampled twice in the field) within our dataset. A maternity analysis was performed to verify the maternity of all twins and to allow for an evaluation of marker power via computed exclusion probabilities. Identity analyses, maternity analyses, and the calculations of exclusion probabilities, allele frequencies, null allele frequencies and heterozygosities (observed and expected) were conducted using Cervus v.3.0.3 (Kalinowski et al., 2007; Marshall et al., 1998; Slate et al., 2000).

2.4. Relatedness analysis simulation

Prior to the relatedness analysis of the empirical twin dataset, a simulation was performed to provide an assessment of different estimators of relatedness coefficients (r_{xy} hereafter), as well as expected means and variances for relationship categories (as described in Ivy et al., 2009). The simulation determined the most appropriate r_{xy} estimator for our dataset and the research questions addressed here.

Given the allelic frequencies within the population (based on $n = 94$), 2,000 individual genotypes were simulated. From the simulated genotypes, 1000 dyads (or comparisons between two simulated individuals) were drawn for four relationship categories (unrelated, half-siblings, full-siblings and parent-offspring) and r_{xy} was calculated for each dyad within each relationship category. The calculation of r_{xy} for each dyad, within the four relationship categories listed above, was performed using six separate estimators (Li et al., 1993; Lynch, 1988; Lynch and Ritland, 1999; Milligan, 2003; Queller and Goodnight, 1989; Ritland, 1996; Wang, 2002) as described in Wang (2011). The estimator with the lowest variance across the relationship categories was chosen for subsequent analyses. Confidence intervals (95%) for the estimation of r_{xy} for the twin groups were calculated using bootstrapping (1,000 samples). All simulations and calculations of r_{xy} for the empirical dataset (including estimation of 95% CI) were conducted using Coancestry v.1.0.0.0 (Wang, 2011).

Initially, relationship assignment between twin siblings was based on the calculated r_{xy} . However, in a few instances, discerning full and half-siblings was challenging since these relationship categories overlapped considerably in their r_{xy} distributions. Therefore, a statistical approach for testing relationship hypotheses via the calculation of likelihood ratios of putative over alternative relationships was employed using ML-Relate (Kalinowski et al., 2006). For each twin sibling pair, we tested one of the following three hypotheses: full (putative) vs. half sibling (alternative), half (putative) vs. full sibling (alternative) or unrelated (putative) vs. half-siblings (alternative). The decision of which hypothesis to test relied on the r_{xy} value obtained for the dyad. In these specific tests, 10,000 genotypes for the alternative relationships were simulated for the significance (p-value)

Table 2

Microsatellite markers used to genotype Antarctic fur seals ($n = 94$ randomly sampled pups) from Cape Shirreff, Livingston Island, Antarctica.

Locus	Source	Species	Repeat Motif	Tm	K	Allele S	Ho	He	p (HW)	Freq (Null)	Missing	Error
Aa4 t	Gemmell et al. (1997)	<i>Arctocephalus australis</i>	not available	55	7	209–225	0.75	0.75	0.5635	–0.0048	0.02	0.02
Ag10 t	Hoffman et al. (2008)	<i>Arctocephalus gazella</i>	(AC) ₁₃	50	7	185–201	0.796	0.796	0.8543	–0.0036	0.01	0
Ag4 t	Hoffman et al. (2008)	<i>Arctocephalus gazella</i>	(GT) ₆ GA(GT) ₁₂	60	23	154–230	0.904	0.908	0.5679	0.0005	0	0
Ag7 t	Hoffman et al. (2008)	<i>Arctocephalus gazella</i>	(GT) ₈ AT(GT) ₁₃	52	8	125–143	0.734	0.778	0.7898	0.0273	0	0
Agaz8t	Hoffman (2009)	<i>Arctocephalus gazella</i>	(AC) ₂₂	52	17	140–190	0.915	0.88	0.5199	–0.0241	0	0
Agaz9t	Hoffman (2009)	<i>Arctocephalus gazella</i>	(GT) ₁₇	50	10	197–219	0.764	0.801	0.2586	0.0189	0.05	0
Hg3.7 t	Gemmell et al. (1997)	<i>Halichoerus grypus</i>	(CT) ₁₀ (CA) ₅ CT(CA) ₁₅	50	12	384–412	0.862	0.845	0.7289	–0.0127	0	0
HI-4 t	Davis et al. (2002)	<i>Hydrurga leptonyx</i>	(GT) ₁₂	52	4	127–131	0.596	0.559	0.9111	–0.0411	0	0
Lc-28 t	Davis et al. (2002)	<i>Lobodon carcinophaga</i>	(GT) ₁₁	58	12	135–171	0.828	0.855	0.6799	0.0134	0.01	0.01
M2B t	Hoelzel et al. (1999)	<i>Mirounga angustirostris</i>	not available	56	9	152–188	0.849	0.844	0.831	–0.0032	0.01	0
Pvc29	Coltman et al. (1996)	<i>Phoca vitulina</i>	not available	52	15	102–146	0.883	0.869	0.8315	–0.0119	0	0.02
Pvc78	Coltman et al. (1996)	<i>Phoca vitulina</i>	(AC) ₁₅	55	9	135–157	0.819	0.818	0.9803	–0.004	0	0
ZcCgDh1.8 t	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(GT) ₁₄ (GC) ₂ (GT) ₈	60	8	155–179	0.787	0.771	0.9081	–0.0148	0	0
ZcCgDh4.7 t	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(GT) ₁₆ (GA) ₁₅	60	12	253–277	0.862	0.85	0.9556	–0.0095	0	0
ZcCgDh48 t	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(TC) ₉ (AC) ₁₄	55	9	258–276	0.581	0.652	0.058	0.0369	0.01	0
ZcCgDh5.8	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(GT) ₂₁	60	14	314–344	0.851	0.866	0.8408	0.0071	0	0
ZcCgDh7tg t	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(TG) ₁₀ (AG) ₁₉	55	16	268–310	0.883	0.888	0.0581	0.0002	0	0
ZcCgDhB.14 t	Hernandez-Velazquez et al. (2005)	<i>Zalophus californianus</i>	(TGGA) ₄ GC(GATC) ₆	60	6	230–258	0.777	0.763	0.5029	–0.011	0	0

Table content: marker names (a “t” after marker name indicates use of a 7 bp tail: GTTTCCT on 5’ of reverse primer), literature source (source), species in which markers were developed (species), repeat motif (when available), annealing temperatures (Tm), number of alleles (K) and allele sizes (Allele S), number of observed heterozygotes (Ho), number of expected heterozygotes (He), Hardy-Weinberg p values (p HW), frequency of null alleles (Freq Null), marker missing data rate (Missing), marker error rate (Error).

estimation. At $p < 0.05$ we accepted the putative relationship over the alternative.

The twinning rate at Cape Shirreff was estimated as the number of genetically confirmed twin births out of the total number of pups born, counted on the US-AMLR study area during the field seasons when the samples were collected.

3. Results

3.1. Genetic marker assessment

The 18 microsatellite markers used in this study averaged 11.71 alleles per locus (range 4 to 23 alleles per locus) and the mean expected heterozygosity (H_E) was 0.81 ($n = 94$). Most microsatellite markers were in agreement with Hardy-Weinberg equilibrium (HWE) expectations. One locus (ZcGgDh48) presented a possible heterozygote deficit ($p = 0.0126$). However, there was no indication of null alleles and the deviation from HWE lost its significance after a Bonferroni correction. Both error rate and missing data per locus were incorporated into all calculations for r_{xy} . No indication of linkage disequilibrium was detected among the loci (153 pair-wise comparisons).

A 0.3% error rate was estimated for the entire dataset based upon replication of PCR amplifications. In all cases, errors in calling alleles were due to weak amplification of a second allele (homozygote call versus a heterozygote call) rather than a complete miscall for that individual at a given locus. The mean proportion of individuals genotyped was 0.9914. All missing data occurred in samples used to estimate population allelic frequencies, and not in the putative twin cases. For estimations of error and missing data rate per locus refer to Table 2.

The expected combined paternity exclusion probability (P_E) calculated using Cervus was 0.9999, indicating high power achieved by the microsatellite marker panel in parentage analysis. This conferred reliability for the genetic analysis of the putative twins as we were able to confidently verify whether they were born to a single mother.

3.2. Relatedness analysis simulation

The relatedness simulation results showed a strong correlation among the 6 relatedness estimators of r_{xy} for the dataset and they all presented relatively low variances (σ^2 range unrelated = 0.0034–0.0133; σ^2 range half-siblings = 0.018–0.0427; σ^2 range full-siblings = 0.0126–0.0653; σ^2 range parent-offspring = 0.0012–0.0573) within each relationship category. Among the estimators, Milligan's dyadic likelihood estimator (Milligan, 2003) had the least variance for all relationship categories (Table 3). Therefore, it was chosen for the following relatedness analysis of twin groups. The parent-offspring relationship category had the least variance and narrowest r_{xy} distribution (r_{xy} $\mu = 0.51$, $\sigma^2 = 0.001$) followed by the unrelated category (r_{xy} $\mu = 0.04$, $\sigma^2 = 0.003$). Conversely, the half-siblings and full-siblings relationship categories presented broader r_{xy} value distributions, with the observed r_{xy} means for half- and full siblings respectively at 0.25 ($\sigma^2 = 0.011$) and 0.50 ($\sigma^2 = 0.013$), matching expected values for second and first order relatives. The simulation results are displayed as probability density distributions of r_{xy} for each relationship category (Fig. 2).

3.3. Genetic analysis of twins

In six of the eight cases, putative twin pairs were confirmed; i.e., we were unable to exclude the females that were nursing them as mothers (each pup shared at least one allele per locus with its mother; parent-offspring $\mu_{r_{xy}} = 0.52$; $\sigma^2 = 0.0014$). In the two remaining cases (CS3 and CS7, see Table 4 for description) the parentage analysis indicated that one of the pups observed nursing along with its

Table 3

Summary of simulation results ($n = 1,000$ dyads per relationship category) for each relatedness coefficient (r_{xy}) estimator. Population allelic frequencies were obtained from 94 Antarctic fur seal pups randomly sampled at Cape Shirreff, Livingston Island, Antarctica.

	Relationship category			
	Unrelated μ (σ^2)	Half-siblings μ (σ^2)	Full-siblings μ (σ^2)	Parent-Offspring μ (σ^2)
Wang r_{xy}	−0.0070 (0.0120)	0.2433 (0.0122)	0.4969 (0.0135)	0.4946 (0.0032)
Lynch & Li r_{xy}	−0.0066 (0.0133)	0.2453 (0.0129)	0.4973 (0.0129)	0.4936 (0.0045)
Lynch & Rit. r_{xy}	−0.0032 (0.0057)	0.2403 (0.0183)	0.4954 (0.0237)	0.4935 (0.0143)
Ritland r_{xy}	−0.0007 (0.0092)	0.2490 (0.0427)	0.4936 (0.0653)	0.4989 (0.0573)
QG r_{xy}	−0.0062 (0.0124)	0.2449 (0.0130)	0.4951 (0.0136)	0.4933 (0.0048)
Milligan r_{xy}	0.0402 (0.0034)	0.2552 (0.0108)	0.5015 (0.0126)	0.5172 (0.0012)

putative sibling on a female (case CS7 is shown in Fig. 3) was not a pup to that mother, and was adopted. In those cases the putative maternity was excluded based on mismatches at six and seven loci. The relatedness analysis revealed that these pups were not fathered by the same male and were unrelated to their adoptive mother regarding other relationship categories (i.e. second or third order relatives); in both cases, mother-adopted pup $r_{xy} = 0$.

In the six confirmed cases, the twins were dizygotic (DZ). In five cases, the twins were likely full siblings ($\mu r_{xy} = 0.46$, $\sigma^2 = 0.004$) and were born to the same set of parents. In the remaining case, the twins were likely half siblings ($r_{xy} = 0.17$) and this was interpreted as a case of heteropaternality, where the twins were born to a single mother but had different fathers. Refer to Table 4 for the significance of relationship hypothesis testing for all twin sibling dyads.

The twinning rate for the Cape Shirreff fur seal population was estimated at 0.12% (6 twins per 4965 births). The twinning rate across years was 0.15% (3 twins per 2,067 births) for season 2006–07, 0.13% (2 twins per 1513 births) in 2008–09, and 0.07% (1 twin per 1385 births) in 2009–10. The inter-annual variation of twinning rate was not statistically significant (Fisher's exact test, $p = 0.88$). As well, the twinning rates estimated for Cape Shirreff presented above and for South Georgia (0.06%, Hoffman and Forcada, 2009) were not significantly different (Fisher's exact test, $p = 0.07$).

4. Discussion

4.1. Genetic marker assessment and relatedness analysis simulation

The genotypes generated for the samples in this study allowed for confirmation of most putative twin cases (six out of eight) identified in the field. The microsatellite markers were highly effective in maternity assignments, as the expected non-exclusion probability was extremely low. Marker power was evidenced by the maternity exclusion at six and seven loci for the two adopted Antarctic fur seal pups.

The genotyping error rate of 0.3% for our dataset lies within an acceptable range of other reports in the literature (i.e. 0.8% Bonin et al., 2004). However, it has been demonstrated that even a low genotyping error can have a significant effect in parentage analysis. For example, a genotyping error rate of 1% can cause false paternity exclusion of 20% (Hoffman and Amos, 2005b). For that reason, the maternity analysis was also performed using a maximum likelihood approach offered by Cervus v.3.0.3 that can incorporate genotyping errors, null alleles and mutations (Kalinowski et al., 2007). Both maternity assignment methods (exclusionary and maximum likelihood) yielded the same results, reinforcing the high power yielded by the 18 microsatellite markers used in this study.

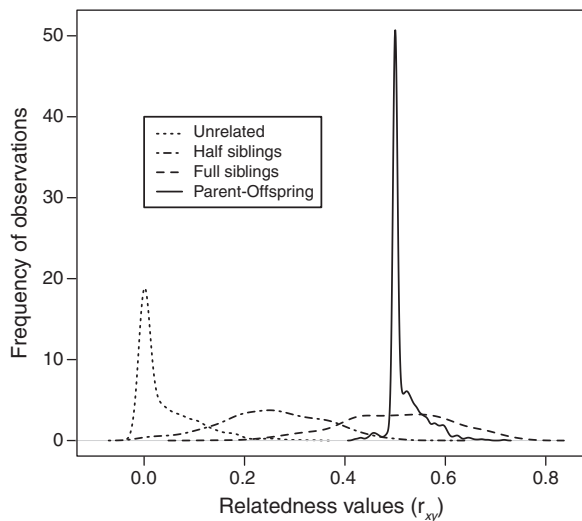


Fig. 2. Probability density of relatedness coefficients (r_{xy}) calculated for 4 relationship categories: unrelated, half siblings, full siblings and parent-offspring ($n = 1,000$ simulated dyads per relationship category). Allelic frequencies were calculated from 94 Antarctic fur seal pups genotyped for 18 microsatellite markers.

The simulation of relatedness analysis was a useful tool for choosing the most appropriate relatedness estimator, as suggested by Csiléry et al. (2006) and Wang (2011). The same analysis also allowed for an assessment of the distribution of relatedness coefficient values (r_{xy}) for each relationship category. The distribution of r_{xy} values confirmed that the parent-offspring category had the lowest variance followed by the unrelated category. Therefore, our analysis would be clearly reliable when used to assign “parent-offspring” or “unrelated” relationships for a pair of individuals. The most challenging assignments were in the full and half sibling categories. Although r_{xy} means for full and half sibling categories matched expectations, they presented higher variance and overlapping distributions. This is not surprising, as it has been demonstrated that 20 microsatellite loci ($H_E = 0.75$) in a vertebrate population are usually

enough to discriminate unrelated from full siblings 97% of the time; however, up to 40 loci maybe required to distinguish between full and half siblings (Blouin et al., 1996). The high heterozygosity ($H_E = 0.81$) found in Antarctic fur seal markers used in this study may confer some ability to distinguish full and half siblings, but the wide confidence intervals (95%) calculated for the twin siblings' r_{xy} revealed that this assignment could benefit from additional loci. In this situation, additional statistical assessments, such as a *a priori* hypothesis testing for relationship categories were an efficient way to assign the most likely relationship for a pair of individuals, as demonstrated by Zeyl's et al. (2009) relatedness study on polar bears (*Ursus maritimus*).

4.2. Twinning in Antarctic fur seals

Our ability to confirm most twinning cases (six out of eight) using genetic analysis indicates that although problematic (Gelatt et al., 2001; Hoffman and Forcada, 2009), field observations of twins in our study area seem to closely reflect actual twinning rates. Therefore, field records of twinning can potentially be used to track changes in the reproductive strategies/ life history of Antarctic fur seals at Cape Shirreff.

Twinning in Antarctic fur seals can be considered rare, with no significant differences between the South Shetland Islands (0.12%) and South Georgia populations (0.06%, Hoffman and Forcada, 2009). This finding is consistent with reports for other pinniped species: 0.2–0.38% for elephant seals, *Mirounga leonina*, (Arnbom et al., 1997; Galimberti and Boitani, 1999; McMahon and Hindell, 2003) and 0.1% in Weddell seals (*Leptonychotes weddellii*; Gelatt et al., 2001). Twinning in pinnipeds is particularly scarce if compared to well-studied groups of mammals such as apes (i.e. chimpanzee, *Pan troglodytes*) with DZ twinning rates estimated at 2.36% (Ely et al., 2006) and ungulates, with twinning rates usually up to 20% (i.e. 2.5–20.7% for European mouflon, *Ovis sp.*, Garel et al., 2005; 9–24% for moose, *Alces alces*, Testa, 2004). A remarkable annual twinning rate of >70% is observed in the saiga antelope, *Saiga tatarica tatarica* (Kühl et al., 2009). The scarceness of twinning in pinnipeds can be explained by the overall high maternal investment for mothers who feed at sea but nurse on land (with the exception of walrus,

Table 4

Values of r_{xy} * estimated for Antarctic fur seal twin groups sampled at Cape Shirreff, Livingston Island, Antarctica.

Case	Individual 1	Individual 2	Milligan's r_{xy}	r_{xy} CI (95%)	p values	Results
CS1	Mother 62444	Twin 1-62445	0.5	0.5	0.5534	
	Mother 62444	Twin 2-62446	0.5856	0.5	0.7356	
	Twin 1-62445	Twin 2-62446	0.5157	0.3466	0.6853	
CS2	Mother 62447	Twin 1-62448	0.5	0.5	0.6508	0.0059
	Mother 62447	Twin 2-62449	0.5	0.5	0.6436	
	Twin 1-62448	Twin 2-62449	0.1753	0	0.3419	
CS3	Mother 62450	Twin 1-62451	0.5377	0.5	0.6699	0.0411
	Mother 62450	Twin 2-62452	0.5422	0.5	0.6323	
	Twin 1-62451	Twin 2-62452	0.376	0.1181	0.6091	
CS4	Mother 62453	Twin 1-62454	0	0	0	0.0001
	Mother 62453	Twin 2-62455	0.5	0.5	0.5711	
	Twin 1-62454	Twin 2-62455	0	0	0	
CS5	Mother 78423	Twin 1-78405	0.6177	0.5	0.7556	0.0125
	Mother 78423	Twin 2-78406	0.5	0.5	0.5936	
	Twin 1-78405	Twin 2-78406	0.4151	0.1336	0.6134	
CS6	Mother 78410	Twin 1-78408	0.5	0.5	0.5451	0
	Mother 78410	Twin 2-78409	0.5001	0.5	0.627	
	Twin 1-78408	Twin 2-78409	0.5124	0.357	0.7775	
CS7	Mother 92464	Twin 1-92546	0.5	0.5	0.5708	0.0016
	Mother 92464	Twin 2-92547	0	0	0.2775	
	Twin 1-92546	Twin 2-92547	0	0	0.3087	
CS8	Mother 92467	Twin 1-92550	0.5	0.5	0.6252	0.0008
	Mother 92467	Twin 2-92551	0.5	0.5	0.6586	
	Twin 1-92550	Twin 2-92551	0.5249	0.2433	0.7146	

* r_{xy} was estimated according to Milligan, 2003 (in Coancestry v. 1.0.0.0 by Wang, 2011). Confidence intervals were generated using 1,000 bootstrap samples. p values represent the significance of the likelihood ratio test calculated for two *a priori* relationships (putative relationship: full sibship, alternative relationship: half sibship); small p values indicate that the putative relationship fits the data significantly better.



Fig. 3. Putative Antarctic fur seal twin case “CS7” identified at Cape Shirreff, Livingston Island, Antarctica. Photo taken in January 2010. Photo credit: Carolina Bonin.

Odobenus rosmarus; Oftedal et al., 1987). In the case of Antarctic fur seals, mothers take foraging trips to sea of two to seven days and spend one or two days nursing their pup ashore. They alternate these activities during the four-month lactation period (Doidge et al., 1986). During their foraging trips to sea fur seal mothers have to gather enough resources for themselves and their nursing pup. This constraint imposes high costs for nursing multiple pups. For example, after observing two female Antarctic fur seals rearing twins to weaning, Doidge (1987) estimated that the energy cost of pup rearing increased by 75% for those females compared to mothers rearing a single pup. Although possible, the rearing of two or more pups incurs a high cost that has influenced the evolution of reproductive strategies in pinnipeds, which rarely give birth to more than a pup.

Other factors related to demographic variables may also determine twinning, and they constitute a basis for interpreting rates of occurrence. These are generally called “maternal effects” and include advanced age, increased parity and matrilineal genetic inheritance (Bortolus et al., 1999; Bulmer, 1970; Hoekstra et al., 2008; Parisi et al., 1983). In humans, twinning rates increase four-fold between the ages of 15 and 37 years, because there is a rise in the level of gonadotropins in females with age. Increased parity also affects the probability of twinning, and although age and parity are highly correlated, their effects are independent of each other (Bulmer, 1970). Pedigree studies in humans (Bulmer, 1970; Lichtenstein et al., 1996; Parisi et al., 1983) and chimpanzees (Ely et al., 2006) also reveal that DZ twinning is a familial trait, mainly inherited maternally. Twinning also has a high recurrence risk at the individual level: a female chimpanzee that has had twins once will have a recurrence risk five times greater than average (Ely et al., 2006). There is limited evidence for the influence of maternal effects in our study site. No twin birth recurrence has been observed at Cape Shirreff. As for age effects, the fur seals in this study were considered of advanced age (range from 11 to 16 years old), given that the female fur seals’ peak in reproduction occurs at 7–9 years of age (Lunn et al., 1994). Thus, although sample size limits our ability to assess the significance of the age effect, our data could support the positive effect of increased parity and age on the twinning probability. As more samples become available, populations of Antarctic fur seals should represent an ideal case for studies on maternal effects of twinning rates in wild pinniped populations.

4.3. Zygosity, heteropaternality and Antarctic fur seal mating strategies

All twin cases confirmed in this study were DZ (fraternal twins). Given our sample size, we expected this result, since MZ twins usually occur at a very low rate in most human (Tong et al., 1997) and chimpanzee populations (Ely et al., 2006). Hoffman and Forcada (2009) reported just one case of MZ twins in Antarctic fur seals, which is also the first confirmed case in pinnipeds.

One case of Antarctic fur seal heteropaternality at South Georgia has been previously reported by Hoffman and Forcada (2009). Our results show that heteropaternality also occurs in the South Shetlands population. One out of the six twinning cases examined here demonstrated that the twin siblings were likely half-siblings, meaning that one of our sampled females conceived from two different males during the same breeding cycle. Heteropaternal DZ twins have been well documented in humans (Bulmer, 1970; Girela et al., 1997; Verma et al., 1992; Wenk et al., 1992) and other primates (Bercovitch et al., 2002; Ely et al., 2006), but their occurrence is always considered rare. The fact that we were able to identify a case of shared paternity within a small sample set (as did Hoffman and Forcada, 2009) indicates that mate infidelity during estrus maybe common in Antarctic fur seals, which has implications for our interpretation of this polygynous mating system.

In summary our study (1) demonstrates the utility of conducting simulations of relatedness analysis for an assessment of marker power and for choosing the most appropriate relatedness estimator, (2) shows that twinning appears to be rare across populations of Antarctic fur seals, indicating the strong constraint likely imposed by the high cost of lactation in this species and in pinnipeds in general and (3) confirms another case of heteropaternality in Antarctic fur seals suggesting that mating infidelity during estrus could be common, which has implications for our understanding of this species mating system.

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