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MICROSATELLITE VARIATION IN NAMIBIAN BROWN HYENAS (*HYAENA BRUNNEA*): POPULATION STRUCTURE AND MATING SYSTEM IMPLICATIONS

JAMES C. KNOWLES,* PETER J. VAN COEVERDEN DE GROOT, INGRID WIESEL, AND PETER T. BOAG

Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada (JCK, PJV, PTB)
Brown Hyena Research Project, P.O. Box 739, Lüderitz, 9000, Namibia (IW)

The genetic structure of brown hyena (*Hyaena brunnea*) populations in any part of their distribution is unknown. Brown hyenas live in clans whose territories and membership change, making nongenetic estimates of population structure and relatedness among individuals difficult to establish. Sixty-one brown hyenas from the west coast of Namibia were genotyped at 10 microsatellite loci designed for the spotted hyena (*Crocuta crocuta*). We found reduced microsatellite variation in brown hyenas compared to spotted hyenas. Using nonhierarchical analyses we detected no major genetic subdivisions across the area sampled in Namibia, but weak differentiation among 2 clans in the southern portion of the range. Females within clans were significantly more related ($r_{wc♀♀} = 0.34 \pm 0.072$ *SE*) than females between clans ($r_{bc♀♀} = 0.022 \pm 0.033$) and than females and males in clans ($r_{wc♀♂} = 0.058 \pm 0.076$). Examination of these data indicates that dominant males were not related to dominant females and that there is multiple paternity within clans.

Key words: brown hyena, dispersal, *Hyaena brunnea*, microsatellites, population genetics, population structure, relatedness

Hyenas are large carnivores that live in Africa and the Indian subcontinent. There are 4 extant species of hyena: the spotted hyena (*Crocuta crocua*), the striped hyena (*Hyaena hyaena*), the brown hyena (*Hyaena brunnea*), and the aardwolf (*Proteles cristata*). Brown hyenas are sexually monomorphic with both sexes approximately the same size as adults. They are primarily nocturnal scavengers, but also are active during the day. In contrast to all other hyenas, brown hyenas have a narrow distribution, living only in a few countries in southern Africa: Botswana, Mozambique, Namibia, South Africa, and Zimbabwe. As of 2008, their conservation status on the *IUCN Red List* is Near Threatened (International Union for the Conservation of Nature [IUCN] 2009) with large populations only in the coastal areas of Namibia, the Kalahari of Botswana, and South Africa (Mills and Hofer 1998).

Most of what is known about brown hyenas is based on studies in the central and southern Kalahari in Botswana. Brown hyenas live in small groups called clans, although adults within a clan do not usually feed or hunt together, and spend most of their time away from other individuals (Owens and Owens 1996). Clans contain 4–14 individuals (Mills 1983), and occupy home ranges of up to 480 km^2 (Mills 1982b). A clan is usually composed of 1 dominant male, 1 dominant female, several other females (suspected of being related to the dominant female), a small number of natal males, and subadults and cubs (Owens and Owens 1996). Unlike the better-known spotted hyena, brown hyena clans are not matriarchal (Holekamp et al. 2007) and the dominant male and female share approximately equal status. Females have been seen to be dominant among other clan individuals in the central Kalahari (Owens and Owens 1996) but not in the southern Kalahari (Mills 1990).

Little is known about the genetic structure of brown hyenas, especially the genetic distinction between clans, although observations of their association and dispersal patterns in the Kalahari suggest that females often live with their natal clans for their entire lives, and some males stay with their natal clans into adulthood (Mills 1990). However, at any given time, many brown hyenas are not living in clans; approximately 65% of brown hyenas live in clans in the southern Kalahari (Mills 1983). Most nomadic individuals are subadult males, which usually leave their natal clans at a young age, with the remainder composed of subadult females as well as adults that have no behavioral clan association (Mills 1982a). Often, nomadic males

* *Correspondent: james.knowles@ualberta.ca*

will integrate with a new clan (Mills 1990). Clan females will mate with either the dominant male in a clan, usually an immigrant (Owens and Owens 1996) or nearby nomadic males (Mills 1982b). The relative reproductive success of the nomads and their role of homogenizing the genetic architecture of the Namibian brown hyena population is unknown.

Although information on the natural history of Kalahari brown hyenas is limited, even less is known about their Namibian counterparts. The 500–1,200 Namibian brown hyenas (Hanssen and Stander 2004) feed opportunistically on reptiles, mammals, and birds, and also eat a significant amount of fruit (Mills and Mills 1978). Individuals that live on the coast primarily feed on Cape fur seals (*Arctocephalus pusillus*—Wiesel 2006). This is atypical for brown hyenas elsewhere, whose diet is largely carrion and fruit rather than live captures (Owens and Owens 1978).

The high variability of microsatellite markers and their recent use in other studies of carnivores makes them promising for elucidating genetic structure in Namibian brown hyenas (Bruford and Wayne 1993). Microsatellites are nuclear DNA loci containing tandem repeats of 1–6 nucleotide base pairs (Queller et al. 1993), with substantial variation in the number of repeats, resulting in high allelic diversity (Bruford and Wayne 1993). Microsatellite loci exhibit Mendelian inheritance and codominance (Queller et al. 1993) and have been used to investigate population genetic structure and dispersal in carnivores such as lions (*Panthera leo*—Gaur et al. 2006; Spong and Creel 2001; Spong et al. 2002), cougars (*Puma concolor*—Anderson et al. 2004), gray wolves (*Canis lupus*—Carmichael et al. 2007; Musiani et al. 2007; Pilot et al. 2006), and striped hyenas (Wagner et al. 2007).

The lack of obvious population structure in the Namibian brown hyena based on field observation suggests that nonhierarchical analyses of microsatellite data (François et al. 2006; Manni et al. 2004) are appropriate for these animals. Nonhierarchical analyses do not require prior knowledge or hypotheses of population structuring, compared to hierarchical analyses such as F_{ST} (Wright 1965) or assignment tests (Waser and Strobeck 1998), where group membership (hierarchy) must be assigned a priori (Miller 2005). A thorough nonhierarchical analysis includes tests for major genetic discontinuities based on pairwise interindividual genetic distances (Manni et al. 2004), followed by identification of putative groups of individuals in Hardy–Weinberg equilibrium, which along with associated geographic data can be used to better understand the ecological and conservation ramifications of population structure (François et al. 2006).

The 1st objective of our study was to evaluate 17 spotted hyena microsatellite loci for use in brown hyenas and to make an initial comparison of microsatellite variability between brown and spotted hyenas. The 2nd objective was to characterize the genetic structure of brown hyenas on the west coast of Namibia, the 1st such characterization for any population of brown hyenas. Further, we sought to evaluate genetic differentiation within and among clans, and determine patterns of relatedness among females and males within clans. Such data would add to our limited understanding of the mating system and dispersal behavior of these animals.

Materials and Methods

Blood and tissue samples: collection and storage.—Samples were collected by the Brown Hyena Research Project from wild captures, roadkill, and animals that died of natural causes and were collected under a research permit, issued and reviewed annually by the Ministry of Environment and Tourism in Namibia, following guidelines approved by the American Society of Mammalogists (Gannon et al. 2007). Initially, 61 tissue samples were collected from the brown hyena population in western Namibia, on the coast of the Namib Desert from 1997 to 2006 (Fig. 1). These included 51 samples collected by the Brown Hyena Research Project with 7 additional samples collected by Andrew Stein (University of Massachusetts, Amherst, Massachusetts), and 3 samples from AfriCat (Otjiwarongo, Namibia). Clan association is inferred for 38 individuals from 11 different clans (9 of which had $\geq$2 individuals sampled) and was based on observed territory occupation and association with other brown hyenas. Skin samples, earplugs, or blood samples were taken from darted, anesthetized brown hyenas. Solid tissue samples ($n = 38$) were stored in dimethylsulfoxide buffer at -20°C. Blood samples ($n = 23$) also were stored at -20°C. Most blood samples consisted of whole blood, although some samples were composed of whole blood without serum. During transportation, tissue samples were stored in ethanol, whereas 100 µl of 1×Tris ethylenediaminetetraacetic acid (1×TrisEDTA) buffer was added to blood aliquots of approximately 1.4 ml. See Appendix I for individual capture locations and sexes.

DNA extraction and microsatellite amplification.—Genomic DNA was extracted from ~150 µl of blood–TrisEDTA mix per sample, following the standard protocol for the QIAamp DNA mini kit (Qiagen, Mississauga, Ontario, Canada). Solid tissue samples also were extracted using a QIAamp kit, following the standard procedure for ~25 mg of tissue. Seventeen loci (7 from Libants et al. [2000] and 10 from Wilhelm et al. [2003]) previously developed from spotted hyenas were assayed for variation in the brown hyena. All primer pairs were prepared for optimization on a LI-COR 4200 sequencer (LI-COR Biosciences, Lincoln, Nebraska), with forward primer sequences preceded by an M13 tag (CACGACGTTGTAAAACGAC).

Each set of primers was evaluated for reliability of amplification in 6 extracted DNA samples. Using a TGradient thermocycler (Biometra, Goettingen, Germany), primer cocktails with 2 concentrations of $MgCl_2$ (1.5 mM and 2.5 mM) were tested using the following polymerase chain reaction conditions: 94°C for 3 min, then 35 cycles of 94°C (denaturing temperature) for 30 s, 55°C (annealing temperature) for 30 s, then 72°C (extension temperature) for 40 s; then a final 7-min extension at 72°C. (See Appendix II for complete polymerase chain reaction cocktail recipes for each locus optimized in this study.)

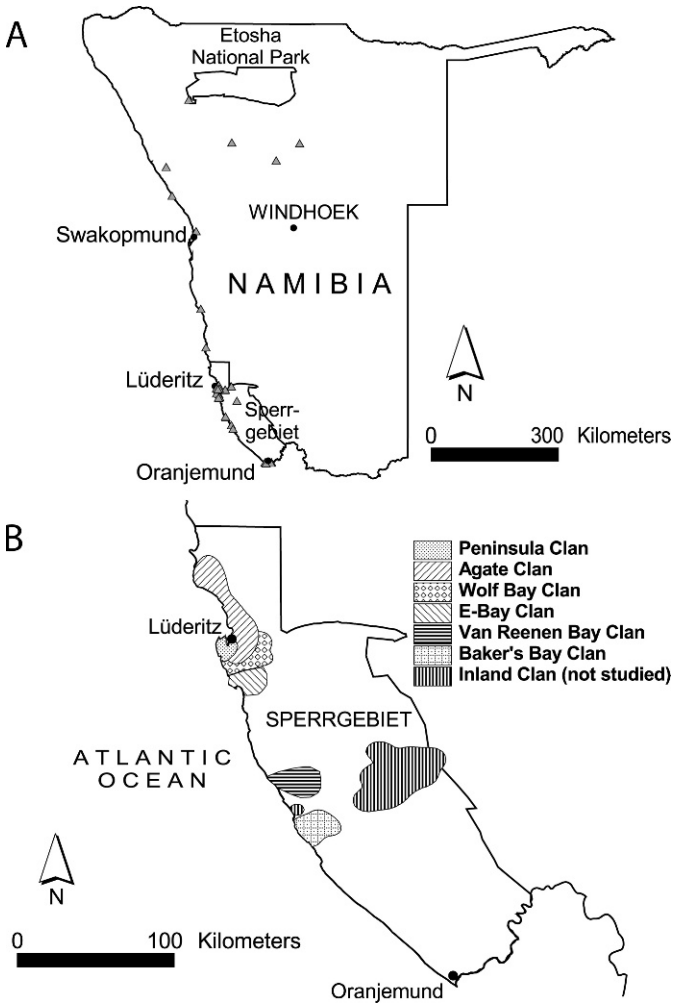

Fig. 1.—Maps of sampling localities and clan distributions. A) Map of Namibia depicting sampling localities of brown hyenas (*Hyaena brunnea*; $n = 61$) used in this study. For the details of sex and location for these samples see Appendix I. B) Map of southwestern tip of Namibia displaying the Sperrgebiet (a biologically diverse area soon to become a national park), which encompasses the territories of several brown hyena (*Hyaena brunnea*) clans, some of which were included in this study: Van Reenen Bay, E-Bay, Wolf Bay, Agate, Peninsula, and Baker's Bay. Clans not included in this map are not well studied, therefore territory size and relative location information are unavailable.

Polymerase chain reaction products were electrophoresed on a LI-COR 4200 automatic sequencer and allelic variation at all reliably amplifying loci was assayed in all samples. Amplicons for optimized loci were sized using the LI-COR IRDye 700 size standard (50–350 base pairs [bp]; LI-COR Biosciences) and GeneIMAGIR 4.05 software (Scanalytics, Rockville, Maryland). Samples that did not amplify initially were reamplified under the same conditions. If, after 2 reamplifications, samples still did not amplify at some loci, another DNA extraction was performed before an additional reamplification. Reextractions and reamplifications for each locus were performed on 5 samples known to be of high quality as a check on laboratory error.

Data analysis.—Variability of microsatellites was compared between brown and spotted hyenas. The number of alleles, observed heterozygosity, and unbiased expected heterozygosity (Nei 1987) were calculated for each locus $\pm$ *SE* using GENALEX version 6 (Peakall and Smouse 2006). To compare heterozygosity between brown and spotted hyenas, a Wilcoxon signed-rank test was used. Because microsatellite loci frequently exhibit reduced variability when amplified in species other than that from which they were developed (Vowles and Amos 2006), we expected to see lower variability in brown hyenas at these loci than was reported for spotted hyenas, and therefore used a 1-sided test to explore this.

Potential genetic differentiation among Namibian brown hyenas was evaluated at a coarse level, then at increasingly finer scales. We 1st conducted a Mantel test (Mantel 1967) where pairwise correlations between interindividual geographic and genetic distances are computed for the entire sample set. A geographic distance matrix between individual hyenas was calculated from capture locations (Appendix I). Because unique geographic coordinates are required for each individual in these and subsequent analyses, some of the latitude and longitude coordinates were modified slightly if animals captured in the same area had been given identical capture locations. Using data from all loci, we calculated nonstandardized pairwise interindividual genetic distances (Smouse and Peakall 1999) among all samples and then the correlation matrix R_{xy} between genetic and geographic distances (km). The matrix's significance was tested against 9,999 permuted matrices using GENALEX.

We used Monmonier's algorithm (Monmonier 1973) to explore for genetic discontinuities in our study population. Using interindividual genetic distances (Smouse and Peakall 1999) and geographic distance matrices, analyses were executed in BARRIER version 2.2 (Manni et al. 2004). In all analyses a single barrier was selected, with strength of each barrier assessed by comparison to a single barrier in 99 bootstrap pseudo-replicates of the data.

Next, we asked if Namibian brown hyenas could be considered a single population in Hardy–Weinberg equilibrium. We used a Bayesian clustering algorithm (TESS version 1.2—François et al. 2006) to search for clusters at Hardy–Weinberg equilibrium using sample genotype and geographic data. TESS output is affected by 2 important parameters: K, the maximum number of clusters the program will attempt to place individuals into, and ψ, an ''interaction parameter'' reflecting the probability that individuals neighboring each other are part of the same cluster. To determine appropriate K and ψ values, our runs varied K from 1 to 10 across $\psi = 0.0$ (which completely ignores spatial data) to $\psi = 1.0$ (which puts significant emphasis on spatial data) at increments of 0.2. Each run consisted of 50,000 ''burn-in'' cycles and 100,000 ''sweeps'' (François et al. 2006). Runs were performed using the *admixture* model, which assumes that individuals may have obtained different parts of their genotypes from different clusters (i.e., the individuals' ancestors could have come from different clusters). The number of clusters was consistently

Table 1.—Variability of optimized spotted hyena (*Crocuta crocuta*) microsatellite loci in brown hyenas (*Hyaena brunnea*) suggesting reduced variability in the brown hyena compared to the spotted hyena at these loci. At all 5 CCROC loci (Wilhelm et al. 2003) and 4 of the 5 CCR loci (Libants et al. 2000) the number of observed alleles is lower in brown hyenas. Also shown are Nei's unbiased expected heterozygosity (H_E) and the observed heterozygosity (H_O—Nei 1987) along with standard errors of these estimates.

Locus	No. alleles observed in 56 brown hyenas	No. alleles observed in spotted hyenas	H_E (brown hyenas)	H_O (brown hyenas)
CCROC01[a]	4	8	0.527	0.482
CCROC02[a]	2	10	0.086	0.089
CCROC05[a]	6	8	0.651	0.481
CCROC07[a]	10	12	0.764	0.679
CCROC09[a]	7	8	0.667	0.527
CCR11[b]	1	2	0	0
CCR12[b]	3	4	0.440	0.500
CCR13[b]	4	4	0.522	0.429
CCR15[b]	2	5	0.329	0.375
CCR16[b]	1	2	0	0
$\bar{X}$ ± SE	4	6.3	0.399 ± 0.090	0.356 ± 0.076

[a] Wilhelm et al. (2003) from 12–46 individuals.
[b] Libants et al. (2000) from 38 individuals.

found to increase with K for ψ values < 0.4, but never exceeded 2 clusters at $\psi \geq 0.4$. This was taken as evidence that setting $K = 3$ was an appropriate setting for $\psi \geq 0.4$ (Chen 2006). Subsequently, for each of $\psi = 0.4, 0.6, \ldots, 1.0$ we performed 50 replicate runs at $K = 3$ to minimize sampling bias due to the stochastic nature of TESS. The run with the highest likelihood value within each set of parameters was considered the best clustering result.

To investigate if there were any detectable genetic patterns at the level of the clan we 1st calculated θ (analogous to F_{ST}, as described by Weir and Cockerham [1984], and henceforth referred to as F_{ST}) among all clans having >1 sampled individual, with 1,000 permutations used to generate confidence intervals using FSTAT version 2.9.3.2 (Goudet 1995). These analyses were executed despite the small numbers of individuals in distinct clans to allow preliminary comparisons between Namibian brown hyenas and other carnivore studies that report F_{ST} statistics. We then conducted a principal coordinates analysis (PCoA) using nonstandardized pairwise interindividual genetic distances (Smouse and Peakall 1999) on the samples that were assigned to specific clans. To confirm male-biased dispersal in this species, we also completed F_{ST} analyses and PCoAs for females alone.

To determine if individuals within a clan were more genetically similar to each other than individuals from different clans, average within-clan relatedness (calculated using MER [Wang 2002]) was compared to average among-clan relatedness using Student's *t*-tests as well as a nonparametric permutation test with 10,000 random re-sorts using the Web tool VassarStats (http://faculty.vassar.edu/lowry/resamp1.html). Nonparametric tests are preferred given the nonindependence of relatedness estimates. To determine if relatedness patterns differed between adult males and females, we completed the same analysis using clan females alone.

Finally, we compared patterns of average relatedness between females within clans with estimates between females and males and between males within clans using a re-sorting analysis of variance with 10,000 random re-sorts with Resampling Stats for Excel (Resampling Stats, Inc., Arlington, Virginia). After randomly re-sorting the data points, a new summed absolute deviation for each re-sorted data set is calculated. The summed absolute deviation from the original data set is compared to the distribution of all randomly re-sorted replicates, and the proportion of replicates with a summed absolute deviation greater than that of the data set is used as a *P*-value. This nonparametric test was done in order to account for nonindependence of pairwise relatedness estimates.

Results

Spotted hyenas are more variable than brown hyenas at microsatellite loci.—Ten of the 17 spotted hyena loci were optimized for amplification of brown hyena microsatellite DNA. The remaining loci could not be amplified reliably in brown hyenas. Of the 10 loci, 2 were monomorphic, 2 were diallelic, and 6 had > 2 alleles, with a maximum of 10 alleles at 1 locus (Table 1). A 10-locus genotype was collected for 61 individuals yielding a mean observed heterozygosity of $H_O = 0.356 \pm 0.076$ *SE* and a mean expected heterozygosity of $H_E = 0.399 \pm 0.090$ (Table 1). Similar to spotted hyenas, the 5 CCROC loci (Wilhelm et al. 2003) were more variable in brown hyenas than the 5 CCR loci (Libants et al. 2000). To compare brown and spotted hyenas, we limited our study set to 56 animals because poor DNA quality meant that 5 individuals amplified at <8 of 10 loci. Our estimate of laboratory error, based on reextraction and re-amplification of 5 samples, was calculated to be ≤5% across all loci.

Variability at the 10 optimized spotted hyena loci appears lower in brown hyenas than in spotted hyenas, with fewer alleles in brown versus spotted hyenas (Table 1). More specifically, the 5 CCROC loci (Wilhelm et al. 2003) that amplified in both species had significantly higher H_E ($H_E \bar{X} = 0.808 \pm 0.036$) and H_O ($H_O \bar{X} = 0.834 \pm 0.069$) across 40

spotted hyenas than our H_E ($H_E \bar{X} = 0.539 \pm 0.119$; 1-sided Wilcoxon signed-rank test, $P = 0.031$) and H_O ($H_O \bar{X} = 0.452 \pm 0.098$; 1-sided Wilcoxon signed-rank test, $P = 0.031$) in 56 brown hyenas. Similarly for the 5 CCR loci (Libants et al. 2000), the H_O ($H_O \bar{X} = 0.659 \pm 0.093$) and H_E ($H_E \bar{X} = 0.597 \pm 0.075$) for these loci in 38 spotted hyenas was significantly greater than our H_O ($H_O \bar{X} = 0.261 \pm 0.108$; 1-sided Wilcoxon signed-rank test, $P = 0.031$) and H_E ($H_E \bar{X} = 0.258 \pm 0.110$; 1-sided Wilcoxon signed-rank test, $P = 0.031$) in the 56 brown hyenas. In all subsequent analyses, both monomorphic loci were excluded.

No obvious population genetic structure in Namibian brown hyenas.—There was a small but significant correlation between interindividual genetic and geographic distance in Namibian brown hyenas: $R_{xy} = 0.244$ ($P < 0.001$ after 9,999 permutations), but no strong genetic barrier separating groups of individuals was detected using BARRIER (Fig. 2). The strongest barrier was between 2 individuals in the northern part of the population (IW1 and IW129), but this received only 52% bootstrap replicate support.

TESS runs at $K = 3$ with the highest likelihood scores revealed only 1 or 2 clusters of individuals at Hardy–Weinberg equilibrium (Fig. 3). At $\psi = 0.4$, a single genetic unit of brown hyenas is supported in all 50 runs (Fig. 3A); however, with increasing ψ, 2 genetic groups are indicated in the runs with highest likelihoods (Figs. 3B and 3C). For $\psi = 0.6$ and $\psi = 0.8$, there is a single split between the 2 clusters, about half-way through the hyenas' range (Fig. 3B). At $\psi = 1.0$ the same split is present, but there is also a 2nd split near the southern end of the range, which causes 1 cluster to be on opposite sides of the other (Fig. 3C). It is noteworthy that although a single genetic unit was recovered by >60% of all replicate runs at all values of ψ, and 2 clusters were detected in >25% of runs at all $\psi > 0.4$, the last result, containing a geographically split genetic cluster, appeared only at $\psi = 1.0$, in 12% of the runs.

More traditional hierarchical analyses support the absence of major genetic structure in this population. For all animals $F_{ST} = 0.118$, with a 95% confidence interval of 0.079–0.169, and no clan was completely distinct from other groups in PCoA (Fig. 4). Eighteen of the 56 individuals were excluded from the F_{ST} analyses and PCoAs because clan data were unavailable for 16 individuals, and the remaining 2 were individuals from clans with only 1 sampled individual. Although most of the Oranjemund individuals are separated from the other hyenas and most individuals from the Peninsula clan appear weakly differentiated, these groups did not include all the individuals from their clans (see circled individuals in Fig. 4). The first 2 PCoA axes accounted for 57% of the observed genetic variation. The PCoA with only clan females showed Peninsula individuals loosely grouping together, but no other clan females grouped together (data not shown). For these females, $F_{ST} = 0.172$ with a 95% confidence interval of 0.105–0.237.

Clan individuals, especially females, are significantly related.—Namibian brown hyenas appear more closely related to individuals in their clan than to individuals from other clans. The mean relatedness of individuals within clans (0.15

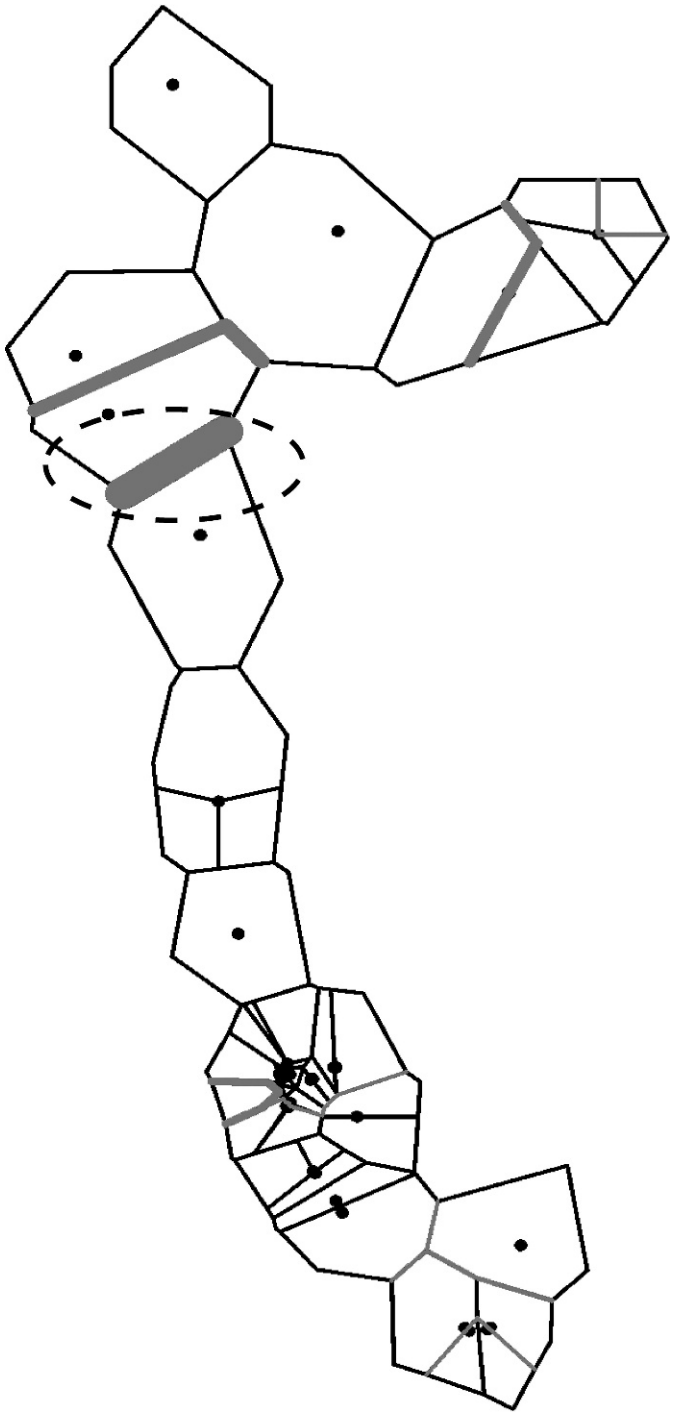

FIG. 2.—Lack of significant population genetic structure in 56 Namibian brown hyenas (*Hyaena brunnea*) as shown by BARRIER output. The strongest barrier is between 2 individuals in the northern part of the population and received about 52% bootstrap support (contained within dashed oval). Individual hyenas are represented by dots, encased in Voronoi tessellation polygons (Manni et al. 2004). Polygons are derived from the distance between individuals, with lines placed in the middle of every pair of individuals that are adjacent to each other. Lines between individuals that are thicker than lines surrounding the external individuals represent barriers selected during analysis. The thicker the barrier line, the more times that barrier was selected among 99 pseudoreplicates.

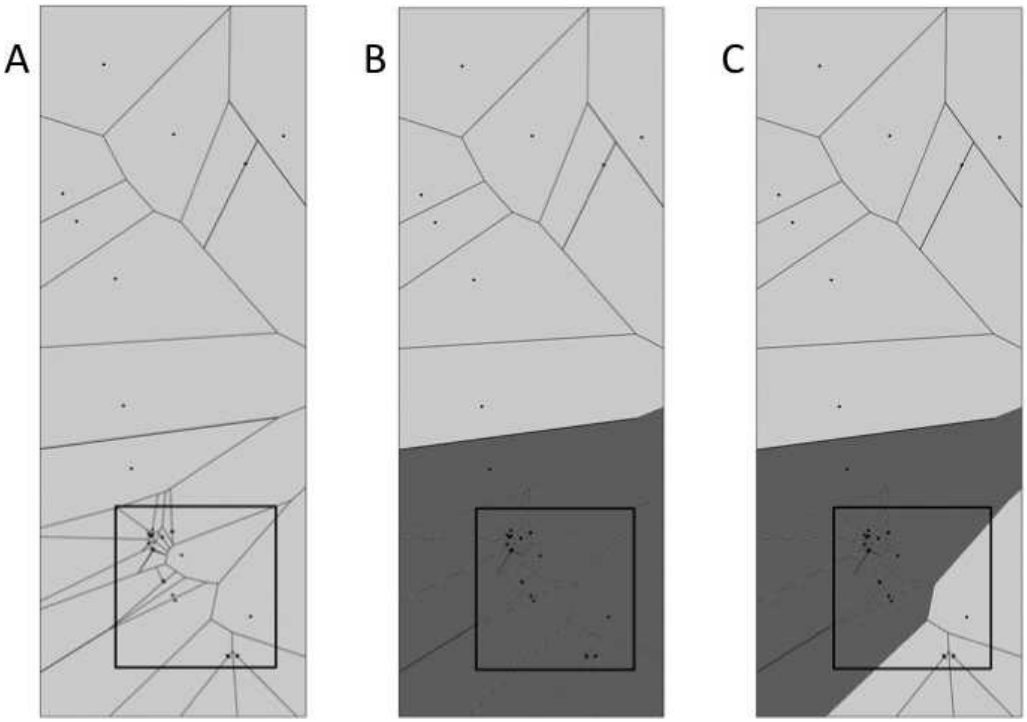

FIG. 3.—Selected clustering pictures from TESS analyses depicting the 3 different results from runs performed at $\psi = 0.4, 0.6, \ldots, 1.0$ and $K = 3$ (50 runs were performed for each ψ value of 0.4, 0.6, 0.8, and 1.0, where ψ is an interaction parameter). The relative placement of the samples (dots) is derived from capture locations, and the surrounding polygons are generated using Voronoi tessellation (François et al. 2006). Individuals of the same shade have been identified in the same genetic cluster. The boxes in the middle of each image surround individuals found within the Sperrgebiet. A) The 1st image is the clustering result of the run with the highest likelihood at $\psi = 0.4$. This image depicts no genetic structure in Namibian brown hyenas (*Hyaena brunnea*). B) The 2nd image is the result of the runs with the highest likelihood at $\psi = 0.6$ and 0.8. This image depicts a genetic split between northern and southern parts of the population. C) The 3rd image is the result of the run with the highest likelihood at $\psi = 1.0$, and shows 1 of the 2 clusters broken up in the middle of the range.

$\pm$ 0.041 *SE*; $n = 77$) was greater than the mean relatedness of individuals between clans (-0.019 ± 0.015, $n = 626$; 2-tailed *t*-test assuming equal variance, $P = 0.00011$; after 10,000 re-sorts, $P = 0.0001$).

This pattern was the same considering females alone, with females from the same clan being more closely related than females between clans. The mean relatedness of females within clans ($r_{wc♀♀} = 0.34 \pm 0.072$; $n = 23$) was significantly greater than mean relatedness of females between clans ($r_{bc♀♀} = 0.022 \pm 0.033$; $n = 130$; 2-tailed *t*-test assuming equal variance, $P = 0.00024$; after 10,000 re-sorts $P = 0.0004$).

Finally, females within a clan appear to be more closely related to each other than they are to males in their clan. The mean relatedness estimate for clan females ($r_{wc♀♀} = 0.34 \pm 0.072$ *SE*) was significantly higher than clan females to clan males ($r_{wc♀♂} = 0.058 \pm 0.076$; $n = 38$), but neither was different from the average relatedness of clan males to same clan males ($r_{wc♂♂} = 0.11 \pm 0.058$; $n = 15$; Fig. 5; analysis of variance with 10,000 re-sorts, $P = 0.0218$, Tukey–Kramer honestly significant difference).

DISCUSSION

Examination of our data suggests that brown hyenas are less genetically variable than spotted hyenas at microsatellite loci, there is no major genetic structuring across the sampled range of Namibian brown hyenas, there is some genetic differentiation among clans, and females within clans are more closely related to each other than to males in the same clan or to females in other clans.

Origin of lower microsatellite variability in brown versus spotted hyenas.—Although the lower variability we report mirrors the lower variation observed in ~350 bp of mitochondrial cytochrome-*b* sequence in brown versus spotted hyenas (Rohland et al. 2005), the origin of this apparent distinction is not clear. Even though recent censuses suggest relatively large numbers of both species ($n > 10{,}000$ spotted hyenas and 5,000–8,000 brown hyenas—Mills and Hofer 1998), Rohland et al. (2005) suggest that a recent bottleneck of brown hyenas may have reduced their genetic variation. Because the microsatellite loci in this study were designed from the spotted hyena alone, the role of an ascertainment bias (Vowles and Amos 2006) cannot be discounted. A strong statement of relative microsatellite diversity in these and other hyenas requires loci of similar repeat motif (type and length) from all study taxa (Van Coeverden de Groot and Boag 2004). The role of a recent bottleneck in shaping genetic diversity in these species can then be more accurately tested.

Lack of major population genetic structure in Namibian brown hyenas.—Although the presence of livestock and game

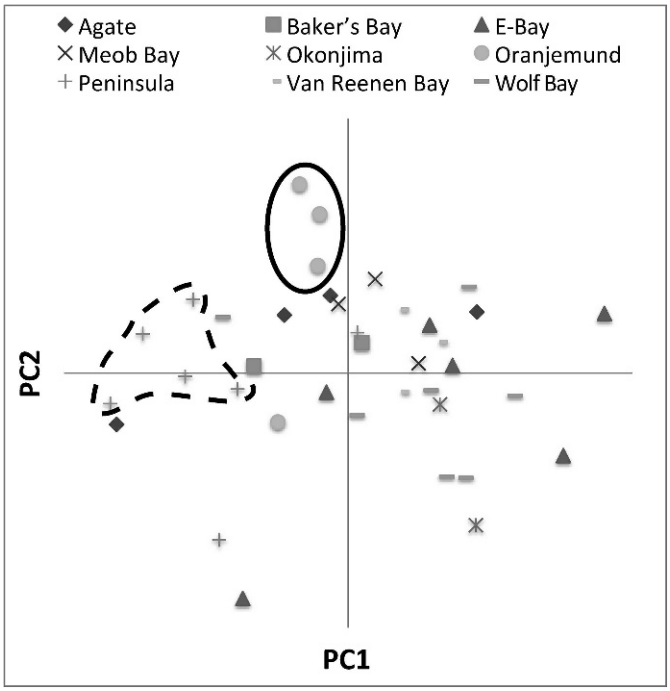

FIG. 4.—A principal coordinates analysis (PCoA) of nonstandardized genetic distances (Smouse and Peakall 1999) shows some clan distinction of the Oranjemund clan (solid oval), and the Peninsula clan (enclosed by a dashed line) compared to other individuals. Known brown hyena (*Hyaena brunnea*) clan affiliations are coded by shape and shading.

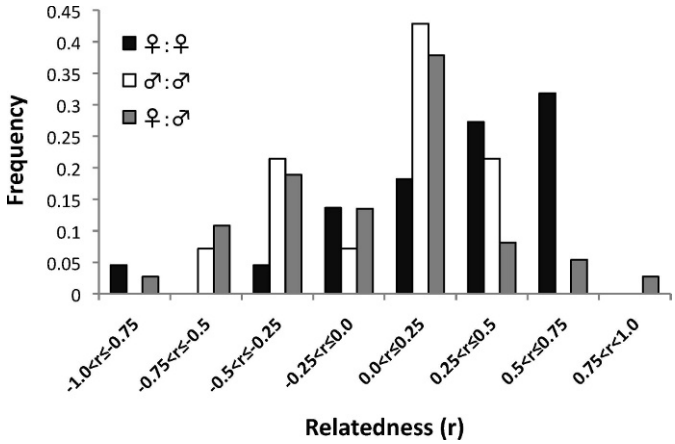

FIG. 5.—Histogram of brown hyena (*Hyaena brunnea*) pairwise relatedness estimates within clans for ♀:♀, ♂:♂, and ♀:♂ pairings showing female-to-female pairs ($r_{wc♀♀}$) as more related than female-to-male ($r_{wc♀♂}$) and male-to-male ($r_{wc♂♂}$) pairs. Average $r_{wc♀♀}$ (0.34 ± 0.072) was significantly higher than average $r_{wc♀♂}$ (0.058 ± 0.076), but neither was different from the average $r_{wc♂♂}$ (0.11 ± 0.058; analysis of variance with 10,000 re-sorts, $P = 0.0218$, Tukey–Kramer honestly significant difference). See text for details.

farming operations between our southern and northern sample clusters led to an expectation of genetic discontinuities across this area, no large genetic break in contemporary Namibian brown hyenas was detected. With $R_{xy} = 0.244$, geographic distance between individuals explains only 6% of the variation in genetic distance among hyenas ($R^2 = 0.0596$). Our study must be considered a preliminary exploration of genetic distance and geographic distance in these animals because the Mantel test does not include any consideration of scale, and our distribution of sampling points is not uniform, with many pairs in the smaller distance classes. Correlations among these latter samples will dominate those among fewer, more distantly separated points leading to inaccurate conclusions over all scales. A larger data set will allow the effect of scale to be investigated using a generalized Mantel analysis (Smouse et al. 1986) or spatial autocorrelation of interindividual genetic distances (Smouse and Peakall 1999).

Although the analyses of interindividual distances (BARRIER; Fig. 2) and the search for groups in Hardy–Weinberg equilibrium (TESS; Fig. 3A) point to the absence of significant genetic discontinuities in the population, both suggest an emerging genetic break in the central portion of our study population. However, there are caveats to the immediate acceptance of these findings. The weak barrier along our north–south axis using interindividual genetic distance (Fig. 2) must be viewed against the properties of the individuals involved and the paucity of samples in this area. The detected barrier was between IW1 (unknown sex) and IW129 (♀), and had 52% replicate support. These animals have complete genotypes with IW129 having a unique allele at the CCR13 locus, which leads to increased pairwise genetic distance whenever it is involved. These 2 have no known clan associations, and, if recent immigrants, may not reflect the genetic associations of local animals. The few samples in this area mean this barrier may represent a sampling artifact. Similarly, although the choice of $\psi = 0.4$ suggests a single population in Hardy–Weinberg equilibrium (Fig. 3A), at all $\psi > 0.4$, distinct northern and southern clusters are indicated in the highest likelihood runs (Figs. 3B and 3C). Although the full impact of ψ can only be explored with more samples, it must be noted that the absence of spatial data (i.e., $\psi = 0.0$—similar to the assumptions of STRUCTURE analyses [Falush et al. 2003; Pritchard et al. 2000]) would have led to stronger conclusions of fine-scale genetic structure in the population.

These findings imply that clans are not significantly differentiated at these 8 microsatellite loci across the range of Namibian hyenas. The southern coastal region of Namibia (Sperrgebiet) contains >6 clans of hyenas (Fig. 1B) and we have sampled >1 individual in 6 of them: Agate, Peninsula, Wolf Bay, E-Bay, Van Reenen Bay, and Baker's Bay. Highly differentiated clans would result in a greater number of groups (K) recovered by TESS; however, only 1 genetic cluster of individuals was identified for all hyenas occupying this area at $\psi = 0.4, \ldots, 0.8$.

Implications for the mating system of Namibian brown hyenas.—Our results allow 2 broad inferences about the mating system of Namibian brown hyenas: dominant males are likely unrelated to dominant females, and there is multiple paternity in clans. Although we do not specifically test the relatedness of dominant females to dominant males we draw

this conclusion from behavioral reports in addition to our data. Brown hyena females tend to live with their natal clans throughout their lives (Owens and Owens 1996), leading to the expectation that many if not all intraclan females should be 1st- or 2nd-order relatives with an $r_{wc♀♀}$ elevated over that expected between nonrelatives. Similarly, because some males live with their natal clan for up to 3 years (Owens and Owens 1996), it is expected that many intraclan males (especially subadults and cubs) will be 1st- and 2nd-order relatives, leading to a higher average $r_{wc\ ♂♂}$. Although the average female-to-female relatedness ($r_{wc♀♀} = 0.34 \pm 0.072$) is not significantly different from $r_{wc♂♂} = 0.11 \pm 0.058$, it is significantly higher than that of clan females to clan males ($r_{wc♀♂} = 0.058 \pm 0.076$). This indicates that 1 of the sampled males in most clans is an immigrant and unrelated to the sampled clan females, which likely include the dominant female. Because it has been observed in the central Kalahari that natal males are always submissive to immigrants (Owens and Owens 1996), we conclude that these immigrants are likely to be dominant. Although the genetic and behavioral evidence provide support for the dominant male and dominant female being unrelated, they do not inform on the reproductive success of the dominant male.

Our findings also indicate that the average clan has multiple sires. If a single immigrant male sired most of the young in a clan, most male-to-male relatedness comparisons would be between 1st- or 2nd-order relatives, as would many of the male-to-female comparisons leading to elevated $r_{wc♂♂}$ and $r_{wc♀♂}$. Although we detail above that this is not the case, examination of our data alone does not definitively distinguish between 2 possible types of multiple paternity in brown hyenas: high mating success of nomads (nonclan males), or short tenure of immigrant males (who have high mating success), both of which would result in multiple paternity within a clan and similar patterns of relatedness therein. Nonetheless, should relatively elevated F_{ST} levels (see below) and significantly lower $r_{wc♀♂}$ (versus $r_{wc♀♀}$) be obtained with larger data sets, this would suggest that nomadic males are achieving relatively little reproductive success.

Our results also support male biased dispersal in Namibian brown hyenas and contrast markedly with recent genetic studies of striped hyenas. In the striped hyena, the adult males within a clan tend to be highly related with mean $r = 0.30$ (8 microsatellite loci—Wagner et al. 2007), whereas our mean male-to-male relatedness ($r_{wc♂♂} = 0.11$, 8 loci) was less. These findings are congruent with observed male-biased dispersal in brown hyenas (Mills 1982a) and female-biased dispersal in striped hyenas (Wagner et al. 2007).

Comparisons with other free-ranging carnivores and the relative role of social structure in shaping within-population genetic differentiation.—When compared to other estimates of within-population genetic structure of free-ranging carnivores, our initial estimate of interclan differentiation ($F_{ST} = 0.118$) is high. North American cougars with no clan structure and suspected male-biased dispersal have comparatively low microsatellite $F_{ST} = 0.03$ (9 loci—Anderson et al. 2004). At the other end of the spectrum of social organization are lions with female harems and groups of males (often brothers) siring cubs for 2–3 years (Pusey and Packard 1987), a system more similar to that of Namibian brown hyenas, which also appear to have low interpride differentiation ($F_{ST} = 0.07$; 14 microsatellite loci—Spong et al. 2002). The immediate conclusion from these studies, that social structure alone does not predict within-population genetic differentiation of large carnivores, is further supported by recent studies of gray wolves demonstrating the secondary role social grouping plays in shaping within-population genetic structure (Carmichael et al. 2007; Musiani et al. 2007; Pilot et al. 2006). At the scale of continental Europe (Pilot et al. 2006) and smaller regions within North America (Carmichael et al. 2007; Musiani et al. 2007), genetic structure in wolves is best predicted by ecological factors such as available prey species and terrain, rather than social structure or geographic distance. These findings are particularly germane because the wolves have a similar social structure to that of Namibian brown hyenas (see Mech and Boitani 2003). Although our small sample size, especially in the northern and more inland Namibian areas, precluded a thorough investigation of the diversifying role of similar ecological factors, our initial BARRIER and TESS results point to the possibility of genetic discontinuity within the middle of the range of the Namibian brown hyenas, the area where much livestock and game farming operations occur. Current land-use patterns are relatively recent, within the last 100 years (Werner 1993), thus perhaps the effects of a relatively recent interruption of genetic exchange on Namibian brown hyena genetic architecture have yet to be detected.

Fine-scale population genetic structure within Namibian brown hyenas.—Even though our F_{ST} estimate is based on small samples sizes for some clans ($n = 2$ for Baker's Bay and Okonjima clans), our multivariate ordination (PCoA) results (Fig. 4) demonstrate that some clans are differentiated from others. This implies that our F_{ST} estimate is not an artifact of small sample size alone. Three of the 4 Oranjemund animals grouped together in the PCoA. These include 2 males and a female, so differentiation of this clan could not be entirely driven by female relatedness within the clan (see below). Also, some differentiation of the Peninsula clan is evident (Fig. 4). Our samples were generally small relative to the potential size of the clan, prohibiting a full picture of clan differentiation. Brown hyena clans consist of up to 14 individuals (Mills 1983), but the most we sampled from any 1 clan was 7 individuals (in both the Peninsula and Wolf Bay clans). Small sample size concerns aside, differences among sets of clan females likely contribute to the genetic separation among clans: the F_{ST} value for females only ($F_{ST} = 0.172$) is greater than that of all animals ($F_{ST} = 0.118$). Although the female-only PCoA did not show increased clustering of clan individuals, this is probably because only a small number of individuals were sampled from each clan. Indeed, the clan showing the most clustering, Peninsula, contained the greatest number of individuals ($n = 5$) after males were excluded. Finally, the potential role of the large proportion of nomads in

reducing genetic differentiation among Namibian brown hyenas requires additional samples to evaluate. Although there are no estimates of dispersal distances for brown hyenas, they have been observed to travel >50 km in a single night while foraging (Mills 1982b), and 1 of the authors (IW) located a deceased, collared Namibian nomad >100 km from where he was initially observed. This is of particular interest because our F_{ST} based on few samples indicates greater interclan differentiation than in other large carnivores.

Trends and implications.—Although PCoA and BARRIER results indicate no major discontinuities across the range of Namibian brown hyenas, the inclusion of spatial data in our Bayesian analyses indicates the possibility of an emerging genetic discontinuity within this population that should be monitored. These results suggest that although some genetic exchange is taking place across the ~1,000 km of our study area, additional sampling might reveal more substantial genetic discontinuities due to conversion of lands in the central area to livestock or game farming.

The multiple paternity of clans observed in Namibian brown hyenas also is likely to occur in the central and southern Kalahari populations. Immigrant males in the central Kalahari have short tenures of approximately 2 years (Owens and Owens 1984) and brown hyena clan females are known to mate with immigrant and nomadic males (Mills 1982b; Owens and Owens 1996). As with Namibian brown hyenas, the relative contributions of nomadic males and dominant, short tenured, successful immigrants await direct paternity testing.

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APPENDIX I

Clan association, sex, and capture location (degrees latitude, longitude) for all animals successfully genotyped in this study. M = male, F = female, U = sex unknown. For animals captured at the same location, a unique location was assigned by modifying latitude or longitude, or both, slightly.

Hyaena brunnea.—Agate clan: F, 26.592139 S, 15.178778 E; M, 26.592139 S, 15.178778 E; F, 26.592139 S, 15.178778 E; F, 26.592139 S, 15.178778 E. Baker's Bay clan: M, 27.610331 S, 15.498582 E; M, 27.699923 S, 15.541343 E; U, 21.770944 S, 13.98925 E. E-Bay clan: F, 26.8991 S, 15.192067 E; M, 26.8991 S, 15.192067 E; F, 26.886333 S, 15.197435 E; M, 26.908633 S, 15.1713 E; F, 26.91795 S, 15.1859 E; F, 26.916333 S, 15.183472 E. Meob Bay clan: F, 24.647306 S, 14.721361 E; M, 24.647306 S, 14.721361 E; F, 24.647306 S, 14.721361 E. Okonjima clan: U, 20.870371 S, 16.643223 E; M, 20.870371 S, 16.643223 E. Oranjemund clan: M, 28.552056 S, 16.522444 E; M, 28.555861 S, 16.355222 E; M, 28.56825 S, 16.506167 E; F, 28.578056 S, 16.382833 E. Peninsula clan: F, 26.66605 S, 15.126483 E; M, 26.667556 S, 15.123028 E; F, 26.667556 S, 15.123028 E; F, 26.667556 S, 15.123028 E; M, 26.696139 S, 15.156222 E; F, 26.696139 S, 15.156222 E; F, 26.696139 S, 15.156222 E. Swakop River clan: F, 22.67 S, 14.6 E. Van Reenen Bay clan: M, 27.392408 S, 15.349012 E; F, 27.392217 S, 15.349417 E; F, 27.407764 S, 15.362263 E. Wolf Bay clan: M, 26.713389 S, 15.3325 E; F, 26.803415 S, 15.119667 E; M, 26.704913 S, 15.344615 E; F, 26.80465 S, 15.121017 E; F, 26.803415 S, 15.119667 E; M, 26.80465 S, 15.121017 E; M, 26.62055 S, 15.484383 E. Unknown clan association: M, 26.672778 S, 15.193556 E; F, 26.61985 S, 15.499883 E; F, 25.62905 S, 14.8507 E; U, 20.436533 S, 17.241433 E; M, 20.436533 S, 17.241433 E; M, 20.436533 S, 17.241333 E; U, 20.436533 S, 17.241433 E; M, 21.338632 S, 13.773589 E; M, 27.943671 S, 16.725427 E; F, 20.436533 S, 17.241433 E; U, 20.436533 S, 17.241433 E; M, 20.436533 S, 17.241433 E; F, 19.326517 S, 14.4173 E; F, 26.988117 S, 15.638917 E; M, 26.988117 S, 15.638917 E; U, 20.414319 S, 15.512643 E.

Appendix II

Primer sequences, repeat motifs, and required $MgCl_2$ concentration ([$MgCl_2$]) for each locus used in this study. General primer cocktail recipe for 1 sample is as follows: 1 μl of 10× polymerase chain reaction buffer, 0.03 μl of 100 nM forward primer, 0.03 μl of 100 nM reverse primer, 0.03 μl of 100 nM deoxynucleoside triphosphates, 0.5 μl of LI-COR Tag, 0.1 μl of *Taq* polymerase, and either 7.9 μl of double-distilled H_2O (for 1.5 mM $MgCl_2$) or 7.7 μl of double-distilled H_2O (for 2.5 mM $MgCl_2$, which also includes 0.2 μl of 0.5 M $MgCl_2$). Primer cocktail was added to 1 μl of DNA extract for a total reaction volume of ~10.6 μl.

Locus	Forward primer	Reverse primer	Repeat motif	[$MgCl_2$] (mM)
CCROC01	CCTCAATTAGGAACATAAAAAGTG	GAAGGAAGGAAGCAATATGC	$(CA)_{18}$	2.5
CCROC02	GCATGCAGATAATTTGAAGATG	CAAAAAGGAGAAATTTAGCAGA	$(TG)_{20}$	1.5
CCROC05	ACCAGTGATCTGGACTGGGA	AAAAGTAATATGACTGCCAAAAGC	$(CA)_{27}$	2.5
CCROC07	TCCCTCAAGTCACTCGGTGT	TGCTAATGTTCATTGCAGGG	$(GGAA)_6(GAA)_1(GAAA)_{19}$	2.5
CCROC09	CAGAATTAATCCATATCACAAACTGC	TAGGAACCTCTTGCCGTCAG	$(GT)_{19}$	2.5
CCR11	AAAATATGCAGAACTTTTCTAAGTCA	GATCCTGCTTCAGATTCATGC	$(GA)_{13}$	1.5
CCR12	TAATGCTCTACTGTTGCCTTCT	CTCACAATCAGGACTGGCTATA	$(CA)_{13}$	2.5
CCR13	TCTCCGAAGAACAGCAAGA	CTACCCCTCTCACCTCTTCA	$(CA)_{14}$	1.5
CCR15	TCATTGGTTACAGAAAGTTGAATT	AAAGATTACATGGGGAGACGA	$(CA)_{13}$	1.5
CCR16	ATGATCTGTCTATTTTAAATGCAC	GCCTCCATTTTCTTGCTCT	$(CA)_{13}$	2.5