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Chemical fingerprints encode mother-offspring similarity, colony membership, relatedness and genetic quality in fur seals

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Abstract

Chemical communication underpins virtually all aspects of vertebrate social life, yet remains poorly understood due to its highly complex mechanistic basis. We therefore used chemical fingerprinting of skin swabs and genetic analysis to explore the chemical cues that may underlie mother-offspring recognition in colonially breeding Antarctic fur seals. By sampling mother-offspring pairs from two different colonies, using a variety of statistical approaches and genotyping a large panel of microsatellite loci, we show that colony membership, mother-offspring similarity, heterozygosity and genetic relatedness are all chemically encoded. Moreover, chemical similarity between mothers and offspring reflects a combination of genetic and environmental influences, the former partly encoded by substances resembling known pheromones. Our findings reveal the diversity of information contained within chemical fingerprints and have implications for understanding mother-offspring communication, kin recognition and mate choice.

Significance statement

Understanding olfactory communication in natural vertebrate populations requires knowledge of how genes and the environment influence highly complex individual chemical fingerprints. To understand how relevant information is chemically encoded and may feed into mother-offspring recognition, we therefore generated chemical and genetic data for Antarctic fur seal mother-pup pairs. We show that pups are chemically highly similar to their mothers, reflecting a combination of genetic and environmental influences. We also reveal associations between chemical fingerprints and both genetic quality and relatedness, the former correlating positively with substance diversity and the latter encoded mainly by a small subset of substances. Dissecting apart chemical fingerprints to reveal subsets of potential biological relevance has broad implications for understanding vertebrate chemical communication.

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Introduction

The chemical senses are the evolutionarily oldest and arguably most widespread means of interacting with the outside world. Olfaction in particular is fundamental to animal communication, mediating social interactions as varied as territorial behaviour, kin recognition and mate choice (1). Metabolomic tools such as gas chromatography−mass spectrometry (GC−MS) have made it possible to generate individual-specific chemical "fingerprints". By separating compounds and quantifying their relative abundances, these fingerprints provide a wealth of information, even though not all compounds can necessarily be identified. Both volatile and contact cues are potentially hidden within the extreme complexity of chemical profiles, which is why a mechanistic understanding of chemical communication is still lacking in natural vertebrate populations (2).

In particular, "surprisingly little progress" has been made in understanding the link between vertebrate chemical fingerprints and genotype (2). Experimental studies have shown that females of several species are capable of discriminating potential partners based on olfactory cues (3-5). However, very few studies have demonstrated a convincing link between the molecular composition of chemical fingerprints and genetic traits such as heterozygosity (a measure of genetic quality) and relatedness (6-9). These studies were almost exclusively conducted on a captive population of lemurs, a species known for its conspicuous use of scent marking.

A functional understanding of how genotype is chemically encoded also requires knowledge of how many and which types of substances are involved. This is challenging because, especially in natural populations, an individual's mixture of surface chemicals is not only the product of its genotype, but may also be mediated by hormones, the

microbial flora, body condition and environmental factors (2). Thus, analyses based on overall chemical fingerprints may overlook subtle genetic signatures, while they also make little if any headway towards identifying the specific substances involved. A second less appreciated problem is that the modest panels of around 10−15 microsatellite loci typical of most studies may be underpowered to detect genetic associations as they provide relatively imprecise estimates of both heterozygosity and relatedness (10, 11).

In arguably the only study to report a convincing link between chemical fingerprints and genotype in a natural vertebrate population, Leclaire *et. al* (9) used principle component analysis (PCA) to reduce chemical complexity. They identified a principle component in kittiwakes that correlated significantly with heterozygosity in both sexes and another that correlated with relatedness but only in adult males. However, PCA iteratively maximises the explained variance per component instead of seeking to capture the underlying structure and dimensionality of the data, which makes the resulting components hard to interpret (12). A better approach could be factor analysis (FA), a method from the field of psychology that estimates the latent variable structure of a dataset by dividing the total variability into that common to variables and a residual value unique to each variable (13). Statistical developments that allow factor analysis to be applied to data with more variables than observations (14) have only recently made this approach amenable to studying chemical fingerprints.

Pinnipeds are an important group of marine mammals which provide an unusual opportunity to reveal insights into the basis of chemical communication. Studies of Steller's sea lions and harbour seals have revealed a large repertoire of functional olfactory receptor genes (15) and remarkably high olfactory sensitivity (16) respectively.

Individuals of many pinniped species also have a strong musky smell that has been attributed to secretions of facial sebaceous and apocrine glands (17). These glands are known to hypertrophy during the mating season in at least two species (18), suggesting that olfactory cues may be particularly important during the reproductive phase of the life cycle.

Females of many otariid species breed in dense colonies and alternate lactation ashore with foraging trips at sea, necessitating accurate mechanisms for offspring localisation and recognition (19). Although they use a combination of geographical, visual, auditory and olfactory cues to find and recognise their pups (19), olfactory recognition is particularly important as females of many species accept or reject pups based on naso-nasal inspection (20, 21). Furthermore, a recent experiment on Australian sea lions (22) suggests that female pinnipeds are capable of discriminating filial from nonfilial pups using olfaction in the absence of other cues.

Antarctic fur seals (*Arctocephalus gazella*) provide a highly tractable model system for studying the importance of chemical cues in a free-ranging marine mammal. On Bird Island, South Georgia (Southwest Atlantic), a colony of fur seals has been studied intensively for over two decades (23). In this species, olfaction is known to be important for the close-range recognition of pups (20). However, females also show active mate choice for males who are both heterozygous and unrelated to themselves (24), raising the possibility that chemical cues might be involved not only in mother-offspring recognition, but also in mate choice.

Here, we combined GC−MS fingerprinting of skin swabs and genetic analysis to explore the chemical basis by which Antarctic fur seal mothers may recognise their pups.

As females of this species appear capable of choosing males based on heterozygosity and relatedness, we hypothesised that genotype should be chemically encoded and that this could provide a mechanism by which females could identify their pups. We therefore sampled mother-offspring pairs from two discrete but genetically indistinguishable colonies (see "colony differences" in the Results), which in principle allows genetically encoded substances to be disentangled from those influenced by environmental differences between colonies. We also deployed over 40 microsatellite loci to enhance the power to detect associations between chemical fingerprints and genotype. Finally, we used FA together with a variety of non-parametric approaches to explore the structure of the chemical data and to uncover specific subsets of compounds associated with chemical differences between the colonies, mother-offspring similarity and genetic relatedness.

Results

Chemical and genetic data. Chemical fingerprints and multilocus microsatellite genotypes were obtained for 41 mother-offspring pairs from two breeding colonies at Bird Island, South Georgia (Figure 1). After removing compounds present in the control sample or only in a single individual, the total number of substances in each individual's chemical fingerprint averaged 35.9 and did not differ significantly between mothers and offspring (paired *t*-test, $t = -0.05$, $P = 0.96$). All of the animals were genotyped at 43 highly polymorphic microsatellite loci, 41 of which did not deviate significantly from HWE in either mothers or offspring after table-wide false discovery rate (FDR) correction and were therefore retained for subsequent analyses (Supplementary table S1). The mother-offspring pairs all had match probabilities of 100% (Supplementary table S2).

Colony differences. Multivariate statistical analysis of the relative proportions of each substance revealed highly significant differences between animals sampled from the two colonies, both overall (Figure 2a; ANOSIM, Global R = 0.57, $P < 0.0001$) and separately for mothers (ANOSIM, Global R = 0.58, $P < 0.0001$) and offspring (ANOSIM, Global R = 0.56, $P < 0.0001$). Bayesian structure analyses yielded the highest average log likelihood value for $K = 1$ in both mothers and pups (Supplementary Figure S1) indicating a lack of population structure. By implication, chemical differences between the colonies appear to reflect environmental influences (see Discussion).

Mother-offspring similarity. Pups were significantly more similar to their mothers in their chemical fingerprints than expected by chance (Figure 2b), both overall (ANOSIM, Global R = 0.67, $P < 0.0001$) and within each of the colonies (Special study beach: ANOSIM, Global R = 0.53, $P < 0.0001$; Freshwater beach: ANOSIM, Global R = 0.45, $P < 0.0001$). Chemical similarities between mothers and offspring could be encoded by shared genes or might simply reflect their spatial proximity. However, we found no relationship between chemical similarity and geographic distance within the special study beach, where pupping locations are recorded to the nearest square metre, either for

mothers (Mantel's $r = 0.008$, $n = 20$, $P = 0.44$) or offspring (Mantel's $r = 0.06$, $n = 20$, $P = 0.31$). This suggests that chemical similarity is not associated with geographic proximity *per se*.

Genotype and overall chemical fingerprints. To determine whether genetic relatedness is reflected in chemical similarity, we tested for an association between pairwise r (see Supplementary table S3 for summary statistics) and Bray-Curtis similarity. A highly significant relationship was obtained when all of the animals were analysed together (Mantel's $r = 0.07$ $n = 82$, $P = 0.005$) but non-independence of both chemical and genetic data for mothers and offspring may introduce pseudoreplication. We therefore repeated the analysis separately for mothers and offspring, finding no significant relationships (mothers, Mantel's $r = 0.06$ $n = 41$, $P = 0.10$; offspring, Mantel's $r = 0.030$ $n = 41$, $P = 0.25$).

To test for a chemical signal of genetic quality, we regressed the number of compounds in an individual's chemical fingerprint, a measure of chemical complexity, on standardised multilocus heterozygosity (sMLH). A significant positive correlation was found in mothers (Figure 3, $F_{1,40} = 5.26$, $P = 0.026$) but not in offspring ($F_{1,40} = 0.50$, $P = 0.483$). The strength of correlation also increased steadily with the number of microsatellites deployed in mothers and to a lesser extent in offspring (Figure 4a). Conversely, the estimation error of the parameter g_2, which quantifies the extent to which heterozygosities are correlated across loci, decreased with increasing marker number (Supplementary figure 2). Overall, g_2 was significantly positive (0.0022, $P = 0.032$ based on 1000 iterations of the dataset) indicating that heterozygosity is correlated across the genome.

Factor analysis. Chemical fingerprints are highly complex and may contain numerous compounds influenced by non-genetic factors. We therefore used principal axis factor analysis (FA) to decompose the multidimensional chemical data into four factors (see Methods for details). Fitting the scores of all four factors together in a generalised linear model (GLM) of maternal heterozygosity, factors 1 and 2 were retained as significant

predictor variables (Table 1a) and together explained almost twice as much deviance as the number of compounds in an individual's chemical fingerprint (23.4% versus 11.9% respectively). A simple GLM of sMLH fitting the sum of the two factors as a single explanatory variable explained roughly the same amount of deviance (23.4%, $F_{1,39}$ = 11.91, P = 0.001). In contrast, none of the factors were significantly associated with offspring heterozygosity.

To test whether any of the factors are also associated with genetic relatedness, we used partial mantel tests to derive the statistical significance of each factor while controlling for the others (see Methods for details). Factor 1 was significantly correlated with relatedness in mothers (Mantel's r = -0.123, n = 41, P = 0.028, Supplementary figure 3) but not in offspring (Mantel's r = 0.024 n = 41, P =0.65). None of the other factors correlated significantly with relatedness in either mothers or offspring. As with the signal of heterozygosity, the strength of association between factor 1 and relatedness increased steadily with marker number (Figure 4b).

We next constructed a GLM to test for differences in the values of each of the four factors between the two colonies (Table 1b). Factors 1, 2 and 3 did not differ significantly whereas factor 4 exhibited highly significant differences between the colonies (Figure 5). Thus, factors 1 and 2 both show correlations with genetic traits as well as overlapping distributions between colonies, factor 3 is not significantly associated with any of the variables we measured, and factor 4 represents substances that discriminate the two colonies and must therefore be environmentally influenced.

Identification of important substances. To identify substances that contribute most strongly towards chemical similarity within mother-offspring pairs, we used the "similarity percentages" routine (SIMPER). Selecting the two most important compounds for each of the 41 mother-offspring pairs, we identified a total of 12 substances (Supplementary table S4a). These yield a much stronger pattern of within-pair mother-offspring similarity (ANOSIM, Global R = 0.68, P < 0.0001) than was obtained for the full dataset. Similarly strong patterns were obtained separately for each

of the colonies (Special study beach: ANOSIM, Global R = 0.53, $P < 0.0001$; Freshwater beach: ANOSIM, Global R = 0.31, $P = 0.001$).

We also used SIMPER to search for substances accounting for most of the chemical dissimilarity between the two colonies. This identified a total of 15 substances (Supplementary table S4b) that collectively yield a much higher global R value (ANOSIM, Global R = 0.77, $P < 0.0001$) than was obtained for all of the chemicals. To identify substances associated with genetic relatedness, we used the BIO-ENV procedure embedded in a bootstrap framework (see Methods for details). We obtained a subset of ten substances (Supplementary table S4c) that consistently occurred within the "best" subsets (i.e. maximising the relationship between chemical similarity and relatedness) over all 10×10^6 bootstrap samples and collectively maximised the relationship between chemical distance and relatedness (Supplementary figure 4). Chemical similarity based on these ten substances was significantly associated with genetic relatedness (Mantel's r = 0.164, n = 41, $P = 0.001$).

Finally, we cross-referenced the three lists of substances to evaluate any potential overlap. Of the 12 compounds carrying the strongest signal of mother-offspring similarity, nine also occurred in the subset of chemicals that differ between the two colonies, implying that they may be influenced by environmental conditions ashore. Remarkably, a further two compounds overlapped with the best subset of chemicals associated with genetic relatedness. The mass spectra and Kovats indices of these substances indicate close resemblance to the known pheromones ethyl-9-hexadecenoate and heptadecanoic acid (see Supplementary table S4cand Discussion).

Discussion

Although mother-offspring recognition is under strong selection in many species, little is known about its chemical basis, particularly in natural populations of non-model organisms. We show that fur seal pups are highly similar to their mothers in their chemical fingerprints and that this similarity is largely encoded by a handful of substances that also carry information about either colony or genotype. Our findings provide intriguing insights into how females could use chemical information to recognise their offspring and may also help to explain how fur seals appear capable of exercising mate choice for heterozygous and unrelated partners (24).

Our study was partly motivated by the discovery that female Australian sea lions can identify their pups using only olfactory cues (22). In most vertebrate species, chemical fingerprints show marked differences by sex, age and / or reproductive status (25), a pattern that is partly reflected in our data as it is only the mother's chemical fingerprints that encode genotype. However, the overall chemical fingerprints of mothers and offspring are still very similar, raising the possibility that self-referent phenotype matching (26) could be used in mother-pup recognition. This is a conceptually simple mechanism by which the own phenotype is a representation or template used for the recognition of relatives. Self-referent phenotype matching has been demonstrated in a variety of mammalian, bird and fish species (27). However, further experimental evidence would be needed to show that mother-offspring recognition in fur seals relies on self-matching rather than social learning. Interestingly, allosuckling rates vary considerably among pinniped species, from 6% in New Zealand sea lions to up to 90% in Hawaiian monk seals, suggesting that mother-pup recognition abilities may vary among species (28). The Antarctic fur seal has one of the lowest observed rates of allosuckling (29), which is consistent with the strong pattern of chemical similarity we find between mothers and their pups.

Although chemical fingerprints are widely assumed to encode genetic traits such as relatedness and individual heterozygosity, only a handful of studies have reported the expected associations. Moreover, chemical profiles typically change with age and

reproductive status (25) and genetic correlations have to our knowledge only been detected in breeding adults (6, 7). Analysing the relationship between heterozygosity and chemical complexity separately for mothers and pups shows a clear correlation that increases with the number of loci for mothers, a pattern that is weak or lacking in pups (Figure 4a). Due to the consistency of our results with the literature, we believe this reflects a genuine functional difference between the chemical fingerprints of mothers and pups.

We also find a marked difference in the way that heterozygosity and relatedness are encoded in chemical fingerprints. Heterozygosity is detectable in the overall fingerprint as it is correlated with the number of chemicals, whereas relatedness is encoded by a small subset of chemicals, whose signal is diluted by analysing the overall chemical fingerprint. The diversity of chemicals reflected in heterozygosity could be the result of genetic polymorphisms in the enzymes involved in the synthesis of semiochemicals (6) but may also be influenced by condition dependent factors (see below). In contrast, it makes sense that genetic relatedness could be encoded by a small subset of chemicals which potentially reflect certain genes, such as the major histocompatibility complex (MHC), a highly polymorphic cluster of immune genes detectable through scent (30, 31).

In natural populations, environmental effects on chemical fingerprints are likely to be particularly strong. The only study of a free-ranging, natural population to have detected an association with genotype used PCA to reduce the dimensionality of the chemical data (9). However, this approach is not ideally suited to detecting such signals because a principal component that explains maximal variance may not necessarily provide an optimal representation of the underlying genotype. We applied PCA to our dataset but obtained no significant correlations between any of the resulting principal components and relatedness, and a weaker signal of heterozygosity than was obtained using FA. This could be due to the so called "simple structure" that is obtained by rotation of the factors within FA (32). This results in each substance loading primarily on a single factor and not on the others, meaning that the factors represent subsets of variables that covary and are therefore likely to have a shared basis such as genes or the environment.

FA was considerably more successful than PCA at detecting patterns relating to genotype within our chemical dataset. Factors 1 and 2 together explained almost twice as much of the deviance in heterozygosity as a simple regression on the number of substances, and relatedness was significantly associated with factor 1 but not with Bray-Curtis similarity based on the overall fingerprints. As each factor mostly represents a subset of the total pool of chemicals, this is consistent with Hurst and Beynon's suggestion that the selective assessment of specific semiochemicals may allow individuals to assess genotype more accurately than from entire chemical fingerprints (2).

It is unclear why factor 1 carries information about both heterozygosity and relatedness while factor 2 correlates only with heterozygosity. One possibility is that heterozygosity and relatedness are to some extent signaled by the same substances, potentially deriving from the MHC. As the substances loading on factor 2 are essentially uncorrelated with those loading on factor 1, we speculate that heterozygosity may influence the chemical fingerprint through two or more different pathways. Factor 1 could thus represent a direct pathway from genes to the chemical fingerprint, whereas factor 2 may represent an indirect pathway where body condition or the microbiome could be possible mediators. Future work will aim to explore these possibilities.

An important strength of our study was a sampling design that facilitated disentangling genetically encoded substances from those influenced by the environment. We found that factors 1 and 2, which both encode some aspect of genotype, did not differ significantly in the distribution of factor scores between the colonies, whereas factor 4, which carried no discernible genetic information, showed a highly significant difference. These differences could either be a result of environmental chemicals that directly contribute towards the profile, or could reflect alterations to the chemical fingerprint caused by different conditions on the beaches (e.g. temperature, wind, solar radiation). We would need to sample more colonies to determine the concrete causes.

Another important aspect of our study design was the unusually high genetic resolution provided by 41 microsatellites. Most studies use around 10−15 loci, which for our dataset was insufficient to detect a significant correlation between maternal heterozygosity and compound richness (Fig. 4a). However, the strength of correlation increased steadily as more microsatellites were deployed until a highly significant relationship was obtained with the full marker panel. Similarly, the error with which the parameter g_2 was estimated from the genetic data decreased steadily with increasing marker number. This is consistent with the suggestion that, as long as heterozygosity is correlated across the genome (as is the case where appreciably inbred individuals are present), increasing the number of markers should improve the estimation accuracy of genome-wide heterozygosity, leading to a strengthening of effect size (10, 33). A similar pattern was also obtained for genetic relatedness suggesting that, if many thousands of genetic markers could be deployed, an even greater proportion of the chemical variance should be explicable by genotype (11).

In many species, heterozygosity is associated with fitness (34). In Antarctic fur seals, multilocus heterozygosity at nine microsatellites is strongly predictive of early survivorship and breeding success in females (35) as well as reproductive success in males (36). Females of this species also appear to exert mate choice based on their partner's genotype (24) but it is unclear how this could be achieved. The discovery that heterozygosity and relatedness are both encoded in mother's chemical fingerprints lends support to the hypothesis that chemical cues could be involved, although unfortunately we were not able to include adult males in this study as they are challenging to capture and sedate. Nevertheless, as male fur seals emit a strong musky odour (17) which has been proposed to attract females during the mating season (37), it seems plausible that genotype could also be encoded in male chemical fingerprints.

In order to explore the extent to which genes and the environment influence mother-offspring similarity, we first attempted to identify the most important substances associated with mother-offspring similarity, colony dissimilarity and genetic relatedness. We obtained relatively small subsets of 12, 15 and ten chemicals respectively. In the case

of mother-offspring similarity and relatedness, these subsets yielded much stronger associations than were obtained for the overall fingerprints. This suggests that SIMPER and BIO-ENV were successful in identifying important chemicals within the total set of 213 substances, although this does not preclude additional chemicals playing a lesser role. It is also noteworthy that as many as ten or more chemicals appear to encode relatedness, given that a single locus is expected to provide little power to distinguish anything other than close relatives (2).

Evaluating the overlap between the subsets of chemicals associated with mother-offspring similarity, colony dissimilarity and genetic relatedness revealed an interesting pattern. Of the top 12 substances accounting for the similarity between mothers and their pups, nine also occurred in the subset of chemicals that showed the greatest differences between the two colonies. Although our analysis is not exhaustive as we focused only on the most important substances, this nevertheless suggests that chemical similarity within mother-offspring pairs is strongly influenced by the local environment. A further two substances also overlapped with the subset of chemicals associated with genetic relatedness, implying that mother-offspring similarity also has a genetic basis. Both of these substances reveal similarity to known pheromones, consistent with the previous suggestion that pheromone-like chemical signals may play an important role in mother-offspring recognition across a variety of taxa (38).

Little is currently known about the specific chemicals that signal genetic relatedness in vertebrates (2). Although we were only able to putatively identify three of the top ten substances encoding relatedness using the NIST database, the mass spectra and Kovats indices of these compounds reveal close resemblance to the known pheromones ethyl 9-hexadecenoate, heptadecanoic acid and ethyl stearate (Supplementary table S4c). According to the pherobase database, all three of these substances are part of the chemical communication system of a variety of different taxa, ranging from bumblebees to badgers. Heptadecanoic acid, for instance, is a known pheromone of 33 different species including 26 vertebrate taxa. However, to act as a pheromone in a given species,

a chemical must meet a number of strict criteria (39), which would require experimental evidence (see below).

Although we captured a large number of substances of varying volatility, we only recovered compounds soluble in ethanol and which could be detected by GC−MS. Extraction with other solvents was not possible due to logistic reasons. Nevertheless, even though our sampling of chemicals is likely to be incomplete, our analyses revealed a number of statistically significant and potentially biologically relevant patterns. In addition, we detected a variety of chemicals that may carry important information. However, as some of the compounds may have been further metabolised after extracting them from the skin (40) we cannot exclude the possibility that some of the putatively identified compounds are in fact breakdown products.

Finally, biologically relevant chemical cues can be transferred in a variety of ways, from volatile substances recognised by olfaction to chemicals that act when two individuals are in physical contact (41). As adult female fur seals and their pups conduct naso-nasal inspections during the recognition procedure (20), it is possible that some of the chemicals may act through contact. To unequivocally determine the biological relevance of the chemicals we have identified as well as their precise mode of action would require behavioural assays in the field. This will be challenging, but our results provide the basis for testable hypotheses on potential chemical signals and the substances involved.

Methods

Study site and field methods. 44 mother-offspring pairs were sampled from two breeding colonies–freshwater beach and special study beach, separated by approximately 200m (Figure 1) on Bird Island, South Georgia (54º 00´ S, 38º 02´ W). Breeding females and their pups were captured and restrained on land using standard methodology (42). Seal capture and restraint were part of annual routine procedures of the Long Term Monitoring and Survey programme of the British Antarctic Survey. We obtained chemical samples by rubbing the cheek, underneath the eye and behind the snout with a sterile cotton wool swab. Each swab was individually preserved in a glass vial in 60% ethanol stored at -20°C. All of the samples were obtained immediately after capture by the same team of two seal scientists at both colonies. Tissue samples for genetic analysis were collected as described by Hoffman *et al*. (43) and stored individually at –20°C in the preservative buffer 20% dimethyl sulphoxide (DMSO) saturated with salt.

Chemical analyses. We first took 1ml of each sample and allowed the ethanol to evaporate at room temperature under a fume hood for a maximum of 12h before resuspending in 50µl dichlormethane for subsequent processing. The samples were then analysed on a gas chromatograph (GC) equipped with a VF-5ms capillary column (30m x 0.25mm ID, DF 0.25, 10m guard column, Varian Inc., Lake Forest, CA, USA) and coupled to a quadrupole mass spectrometer (Focus GC-DSQ MS system, Thermo Electron SPA, Rodano, Italy). A blank sample (control with cotton wool and ethanol) and an alkane mix (C8-C28) were analysed as well. 1µl of each sample was injected into a deactivated glass wool-packed liner at an inlet temperature of 225°C and processed in a splitless mode. Carrier gas (He) flow rate was held at 1.2 ml/min. The GC run was initiated at 60°C for three min then ramped at 10°C/min to 280°C, where it remained for 20 min. The transfer line temperature was set to 280°C and mass spectra were taken in electron ionization mode at 70eV with five scans per second in full-scan mode (50–500 m/z). GC–MS data were processed using the program Xcalibur (Thermo Scientific, Germany). To ensure that the scoring of compounds was as objective as possible, we wrote a custom R script (available on request) that compensated for minor shifts in retention times among chromatograms by maximising the number of shared components

between samples through very small (≤0.03ms) shifts in the retention time. To double-check the reliability of the scoring, approximately 10% of compounds were selected at random and scored by eye.

Genetic analysis. Total genomic DNA was extracted from each sample using a standard phenol-chloroform protocol and genotyped at 43 highly polymorphic microsatellite loci (see Supplementary table S1 for details). These were PCR amplified in eight separate multiplexed reactions using a Type It Kit (Qiagen) as described in Supplementary table S1. The following PCR profile was used: one cycle of five minutes at 94°C; 24 cycles of 30s at 94°C, 90s at T_a°C and 30s at 72°C; and one final cycle of 15 minutes at 72°C (see Supplementary table S1 for T_a). Fluorescently labeled PCR products were then resolved by electrophoresis on an ABI 3730xl capillary sequencer and allele sizes were scored automatically using GeneMarker version 1.95. To ensure high genotype quality, all traces were manually inspected and any incorrect calls were adjusted accordingly.

Genepop (44) was used to calculate observed and expected heterozygosities and to test for deviations from Hardy-Weinberg equilibrium (HWE), separately for mothers and pups, specifying 10,000 dememorizations, 1000 batches and 10,000 iterations per batch. Two loci that deviated from HWE in either mothers or pups after table-wide correction for the false discovery rate (FDR) using Q-value (45) were excluded from subsequent analyses, leaving a total of 41 loci (Supplementary table S1). Because milk stealing is common in fur seals and can lead to errors in the assignment of mother-offspring pairs in the field (29), we used the program Colony version 2.0.5.0 (46) to verify that all of our mother-offspring pairs were genuine. Coancestry version 1.0.1.2 (47) was then used to generate a pairwise relatedness matrix based on Queller and Goodnight's statistic, *r* (Queller & Goodnight 1989). Each individual's heterozygosity was expressed as standardised multilocus heterozygosity (sMLH), which is defined as the total number of heterozygous loci in an individual divided by the sum of average observed heterozygosities in the population over the subset of loci successfully typed in the focal individual (48). The two-locus heterozygosity disequilibrium g_2,which measures the extent to which heterozygosities are correlated across loci, was then computed using the

method of David *et al*. (49). Sensitivity of this estimate to the number of loci was explored by randomly selecting different sized subsets of loci and recalculating g_2 1000 times.

To test for population structure, Bayesian cluster analysis of the microsatellite dataset was implemented using Structure version 2.3.3 (50). Structure uses a maximum likelihood approach to determine the most likely number of genetically distinct clusters in a sample (K) by subdividing the dataset in a way that maximises HWE and minimises LD within the resulting clusters. Separately for mothers and pups, we ran five independent runs for each value of K ranging from 1 to 10 using 1×10^6 MCMC iterations after a burn-in of 1×10^5, specifying the correlated allele frequencies model and assuming admixture. The most likely K was then evaluated using the maximal average value of Ln $P(D)$, a model-choice criterion that estimates the posterior probability of the data.

Statistical analysis framework. Any chemicals appearing in the control sample or present in only one sample were excluded from further analyses, leaving a total of 213 substances. To explore the completeness of our sampling, we estimated the maximum number of substances present in the population using the Michaelis-Menten Function, based on a permutation procedure (9999 iterations). Up to 229 substances might be expected in a larger sample of individuals, suggesting that we have sampled around 95% of all potential substances. Analyses were conducted on the relative proportion of each substance (%) to the total amount of substances (51). We then employed a three-step analytical framework to (i) visualise and statistically analyse overall patterns of chemical fingerprint similarity in relation to breeding colony, mother-offspring pair, relatedness and heterozygosity; (ii) tease out subsets of chemicals containing genotypic and environmental information; and (iii) to identify specific compounds involved. Computer code and documentation are provided as a PDF file written in Rmarkdown (Dataset S1) together with the raw data (Dataset S2).

Overall patterns of chemical similarity. The chemical fingerprint data were visualised using non-metric multidimensional scaling (nMDS) (52) based on a matrix of pairwise

Bray-Curtis similarity values calculated from the log(x+1) transformed data. This approach allows visualisation of a high dimensional chemical similarity space by placing each individual in a 2D scatterplot such that ranked between-individual distances are preserved, points close together representing individuals with relatively high chemical similarity. Differences between *a priori* defined groups (i.e. the breeding colonies and mother-offspring pairs) were then analysed through non-parametric analyses of similarities (ANOSIM) (52) using 99,999 iterations of the dataset. ANOSIM is a permutation test that provides a way to evaluate whether there is a significant difference between two or more groups of sampling units without the need for assumptions concerning data distribution or homoscedasticity. These analyses were implemented in R using the Vegan package (53).

Factor analysis. To dissect apart genetic from environmental components, we performed a principal axis factor analysis (FA) on the chemical data. We employed an oblique rotation technique (promax) which allows the factors to be correlated. This type of rotation was used because it is possible that certain compounds within the chemical fingerprint may encode more than one genetic characteristic, e.g. heterozygosity and relatedness, and could thus be correlated with more than one factor. FA cannot be applied when a dataset has more variables than observations (D>>N) because the covariance matrix is singular and an inverse cannot be computed. We therefore used the function factor.pa.ginv() from the R package HDMD, which uses a generalised inverse matrix (14). An important step in factor analysis is choosing a reasonable number of factors to represent the data (32). As our dataset is complex and contains many zero entries, some common methods like parallel analysis may lead to an impracticably large number of factors. Consequently, we applied two methods for determining the optimal number of factors. First, we used the Bayesian Information Criterion (BIC), which optimises the trade-off between model complexity and model fit, and second we used a scree plot, which visually depicts the drop in the factor eigenvalue course (32, 54). Both criteria suggested four factors.

Generalised linear models. To explore the contributions of each of the four factors towards the signal of heterozygosity, we constructed separate generalised linear models (GLMs) of mother and offspring sMLH in which we fitted all four factors together and specified a Gaussian error structure. We then tested for factors that differ significantly between the two colonies by constructing a GLM with colony as the response variable (modeled using a binomial error structure) and the values of the four factors fitted as predictors. For each GLM, we initially implemented a full model containing all of the predictor variables and then used standard deletion testing procedures based on *F* tests (55) to sequentially remove each term unless doing so significantly reduced the amount of deviance explained.

Partial Mantel tests. To test for associations between each of the factors and genetic relatedness, we used the relatedness matrix based on all 41 loci as the response variable and fitted as predictor variables matrices of pairwise similarity at each of the four factors using a Partial Mantel test implemented in the ecodist package (56). This randomises the rows and columns of one dissimilarity matrix while leaving the others unpermuted. Separate models were constructed for mothers and offspring, each using 10,000 permutations of the dataset. Finally, we computed the Spearman rank correlation (Mantel's *r*) and two-tailed *P*-value for the association between relatedness and a factor matrix given the other factors as covariates.

Identification of chemicals. We next attempted to identify specific chemicals associated with breeding colony, mother-offspring similarity and genetic relatedness. First, we assessed the contributions of specific substances to the similarity within groups, using the "similarity percentages" routine (SIMPER) (57). This decomposes all Bray-Curtis similarities within a group into percentage contributions from each compound, listing the compounds in decreasing order of importance. As groups, we specified (i) the two breeding colonies; and (ii) the 41 different mother-offspring pairs.

Second, to explore the contributions of individual chemicals to the signal of genetic relatedness, a continuously distributed variable, we used the BIO-ENV procedure (57) to

identify the "best" subset of compounds within the chemical abundance matrix that maximises the rank correlation between pairwise Bray-Curtis similarities and relatedness. However, with over 200 different chemicals being present in the chemical data matrix, it seems likely that this approach could yield spurious associations, especially given that some of the chemicals were present only in a few individuals. For this reason, we embedded the BIO-ENV procedure in a bootstrap analysis as follows: (i) we randomly subsampled 20 of the 41 mothers 20,000 times; (ii) for each subsample, we randomly selected 10 chemicals, each 500 times; (iii) for each of the resulting 10×10^6 subsamples, comprising 20 individuals and 10 compounds, we applied the BIO-ENV procedure and saved the compounds present in the best subset. We then summed up the occurrences of every chemical throughout all of the subsets and sorted them in decreasing order to represent their relative importance. The basic assumption of our approach is that random correlations will not be consistent over the different subsamples of individuals and compounds, whereas compounds that genuinely encode relatedness should be recovered consistently across many subsets. This procedure was conducted in R using the bio.env() function in the sinkr package (58).

Identification of putative substances encoding mother-pup similarity, colony differences and relatedness were based on two steps: (i) comparing the mass spectrum of a specific substance with the best match of the NIST library (National Institute of Standards and Technology NIST 2005 and 2008) and (ii) calculating the Kovats Retention Index and comparing this to the literature value (obtained from Pherobase.com and chemspider.com). Kovats Indices (59) were calculated by running a sample of linear alkanes (C8-C28) under the identical GC-MS conditions as described above.

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Author contributions

Designed research: J.I.H., B.A.C.

Performed research: M.A.S., J.I.H., J.F., B.A.C., A.G., L.J.E.-P.

Contributed new reagents or analytic tools: J.I.H., C.M.

Analysed data: M.A.S., J.I.H., B.A.C., A.G.

wrote the paper: J.I.H., M.A.S., B.A.C., C.M., J.F.

Competing financial interests statement

The authors declare no competing financial interests.

Figure legends

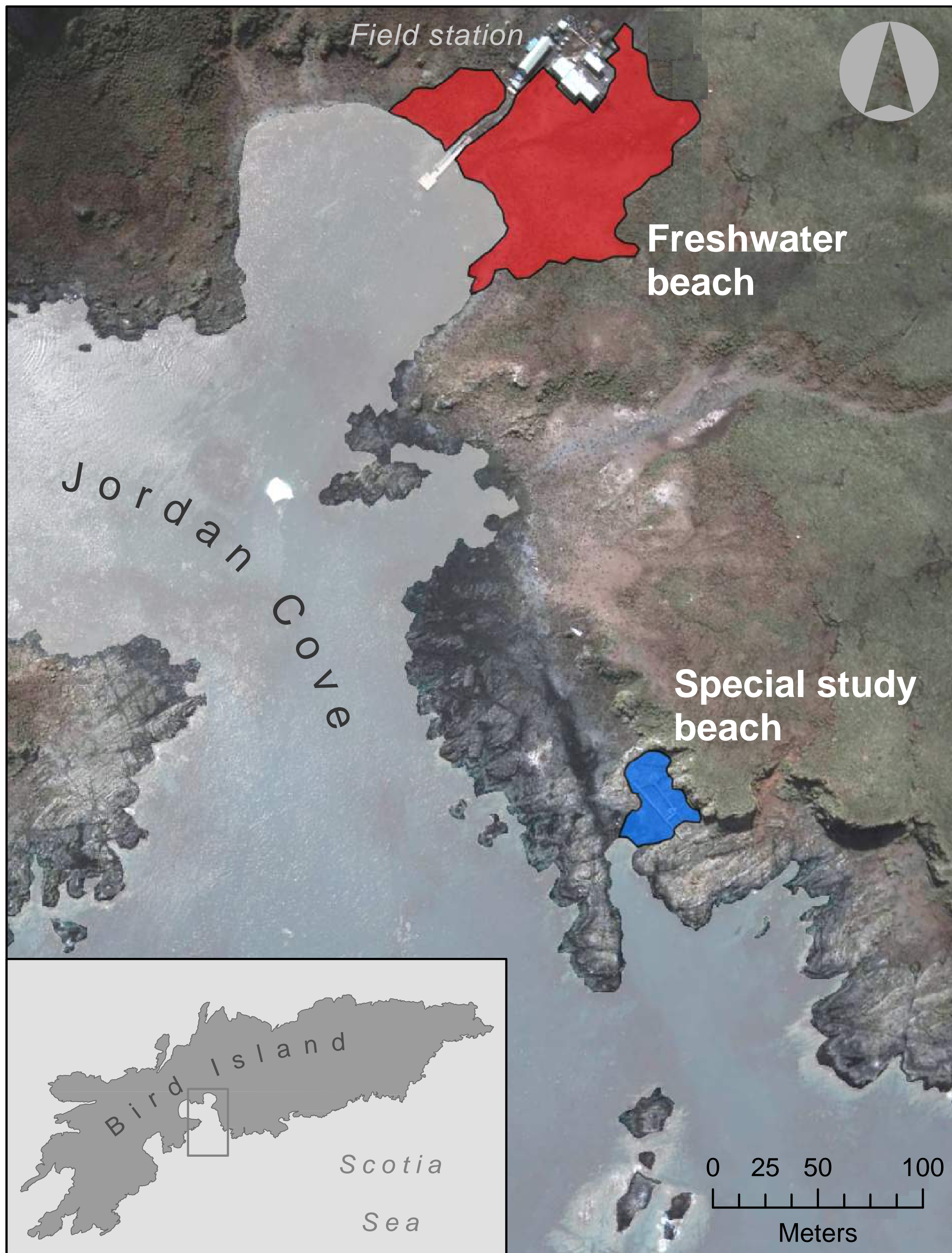
Figure 1 Map of the study area showing the two breeding colonies from which Antarctic fur seal mother-offspring pairs were sampled. The red and blue areas demarcate freshwater beach and the special study beach respectively.

Figure 2 Two dimensional non-metric multidimensional scaling (nMDS) plots of chemical fingerprints of 41 Antarctic fur seal mother offspring pairs. Bray-Curtis similarity values were calculated from standardised and log(x+1) transformed abundance data; (a) colour coded by colony (red points = freshwater beach, blue points = special study beach); (b) plotted by mother-offspring pair, with each pair being denoted by a different symbol / colour combination. The scales of the two axes are arbitrary. The closer the symbols appear on the plot, the more similar the two chemical fingerprints are.

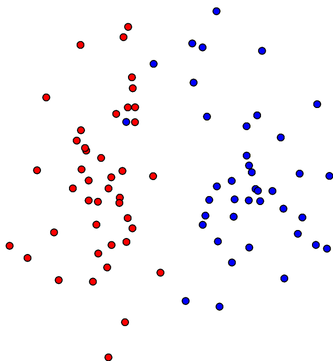
Figure 3 Relationship in mothers between standardised multilocus heterozygosity (sMLH) and the number of compounds in an individual's chemical fingerprint.

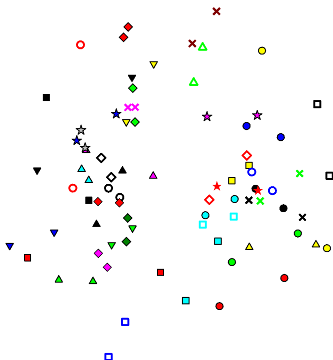
Figure 4 Dependency of the strength of genetic associations on the number of randomly sampled microsatellite loci. Strength of association was quantified as the correlation coefficient (*r*) between (a) sMLH and the number of compounds in an individual's chemical fingerprint (grey symbols) and the sum of an individual's factor 1 and factor 2 values (black symbols), plotted separately for mothers (circles) and offspring (squares); (b) relatedness and Bray-Curtis similarity at the ten best substances in mothers (See Methods for details). Mean ± SE of five resamplings of the data are shown for each point. The dashed lines represent significance thresholds.

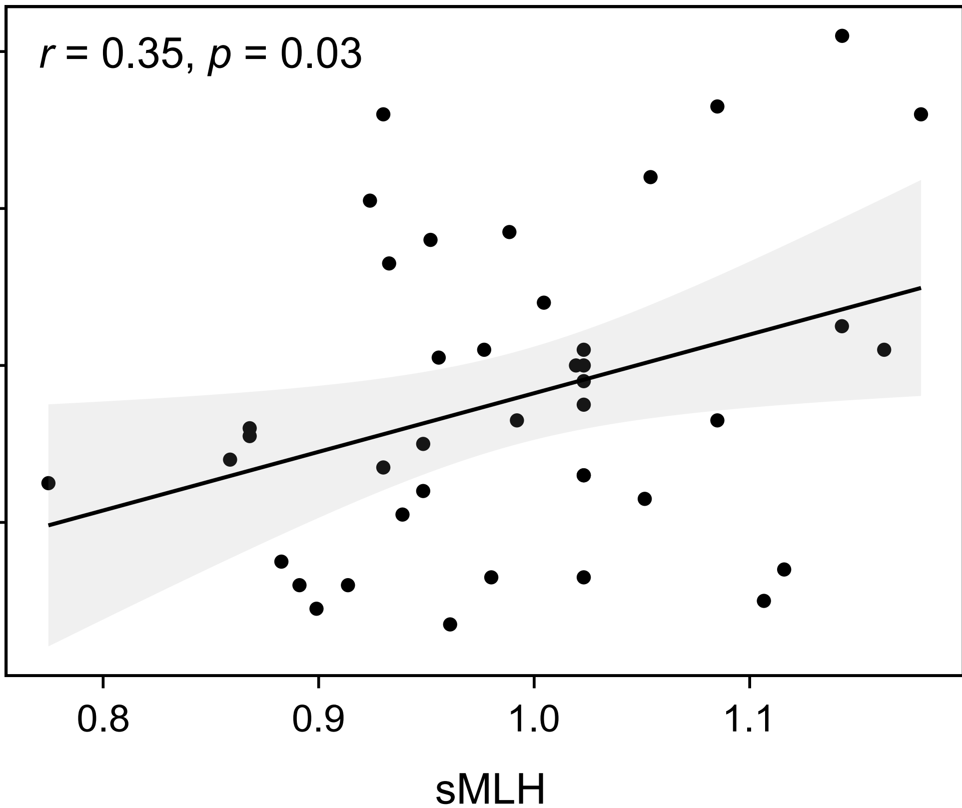
Figure 5 Distribution of factor scores of individuals sampled from the two seal colonies. Factors 1, 2 and 4 are shown in panels (a), (b) and (c) respectively. Freshwater beach is shown in red and the special study beach is shown in blue.

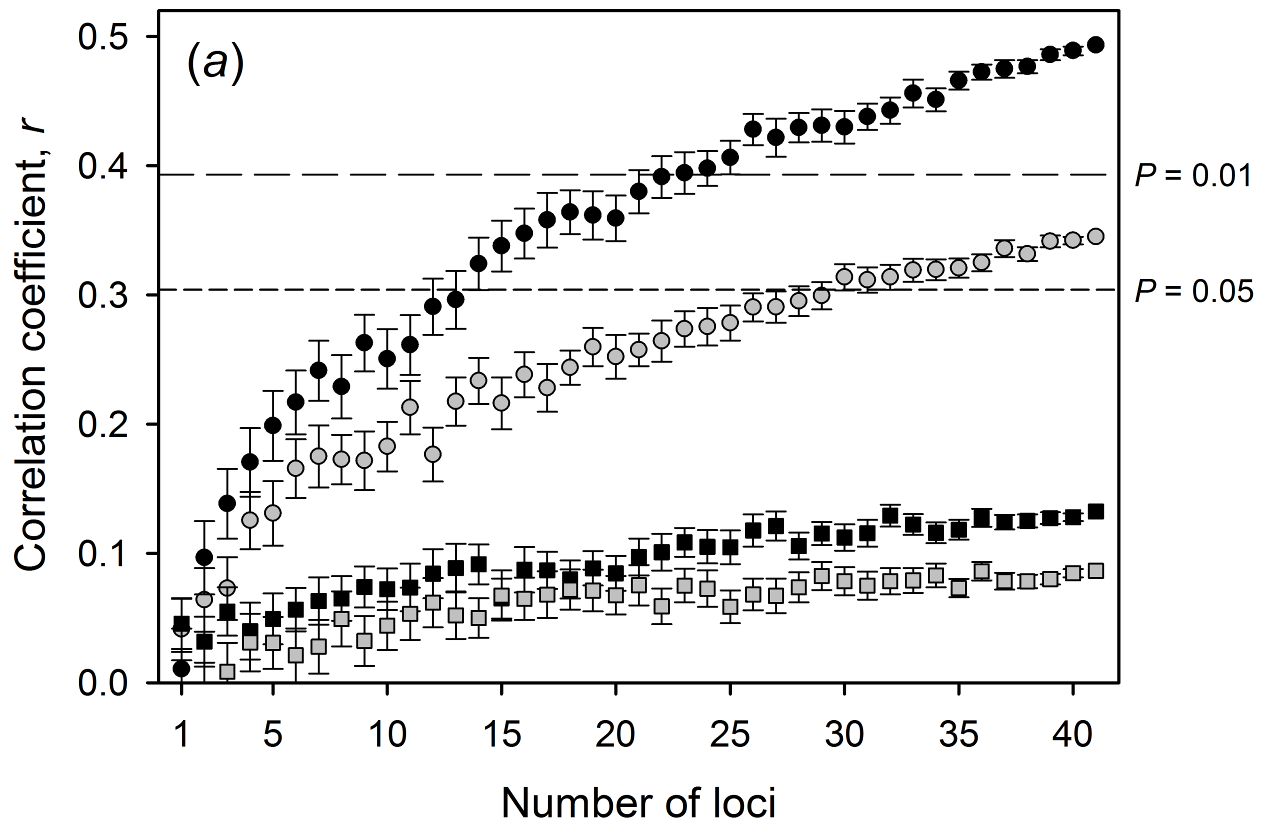


Field station
Freshwater beach
Jordan Cove
Special study beach
Bird Island
Scotia Sea
0 25 50 100
Meters

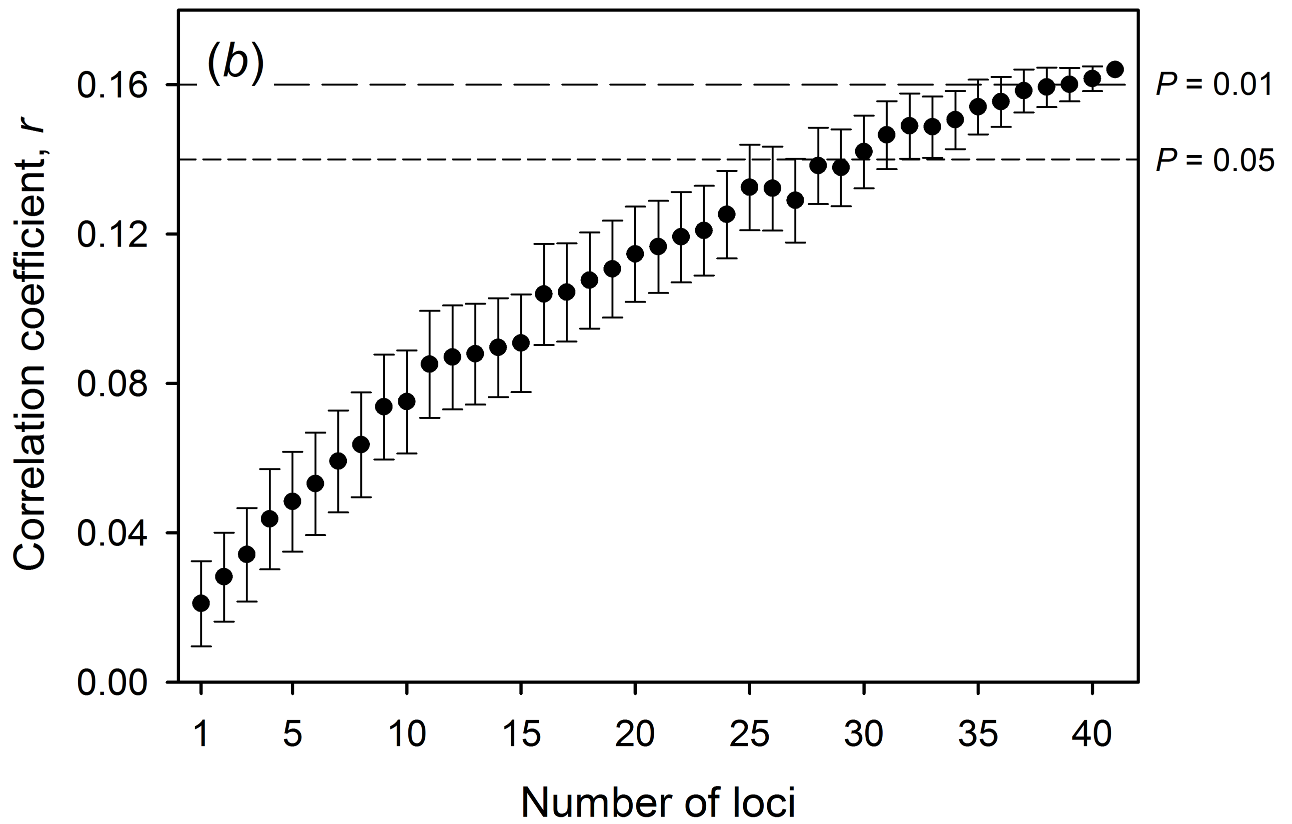

(a)
2D stress: 0.21


(b)
2D stress: 0.21

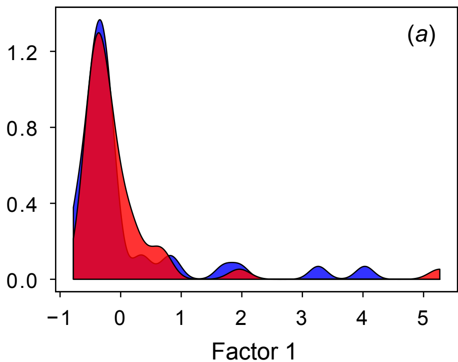

r = 0.35, p = 0.03
Number of compounds
80
60
40
20
0.8
0.9
1.0
1.1
sMLH

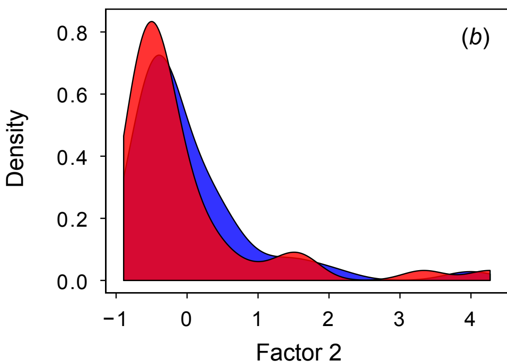


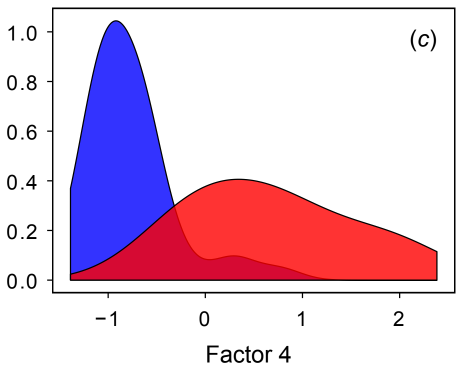
(a)
Correlation coefficient, r
Number of loci
P = 0.01
P = 0.05
0.0
0.1
0.2
0.3
0.4
0.5
1
5
10
15
20
25
30
35
40



(b)
Correlation coefficient, r
Number of loci
P = 0.01
P = 0.05
0.00
0.04
0.08
0.12
0.16
1
5
10
15
20
25
30
35
40


(a)
1.2
0.8
0.4
0.0
−1
0
1
2
3
4
5
Factor 1


(b)
Density
0.8
0.6
0.4
0.2
0.0
−1
0
1
2
3
4
Factor 2


(c)
1.0
0.8
0.6
0.4
0.2
0.0
−1
0
1
2
Factor 4
Special study beach
Freshwater beach

Chemical fingerprints encode mother-offspring similarity, colony membership, relatedness and genetic quality in fur seals

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Supplementary figure legends

Supplementary Figure S1 Results of Bayesian analyses of population structure. Mean ± standard error Ln *P*(*D*) values (filled and open circles, respectively) are shown based on 5 replicates for each value of *k*, the hypothesized number of genetic clusters represented in the data, for (a) mothers and (b) pups.

Supplementary Figure S2 Sensitivity of g_2 to the number of microsatellite loci deployed. Different-sized subsets of loci were each resampled 1000 times and the mean ± SD calculated.

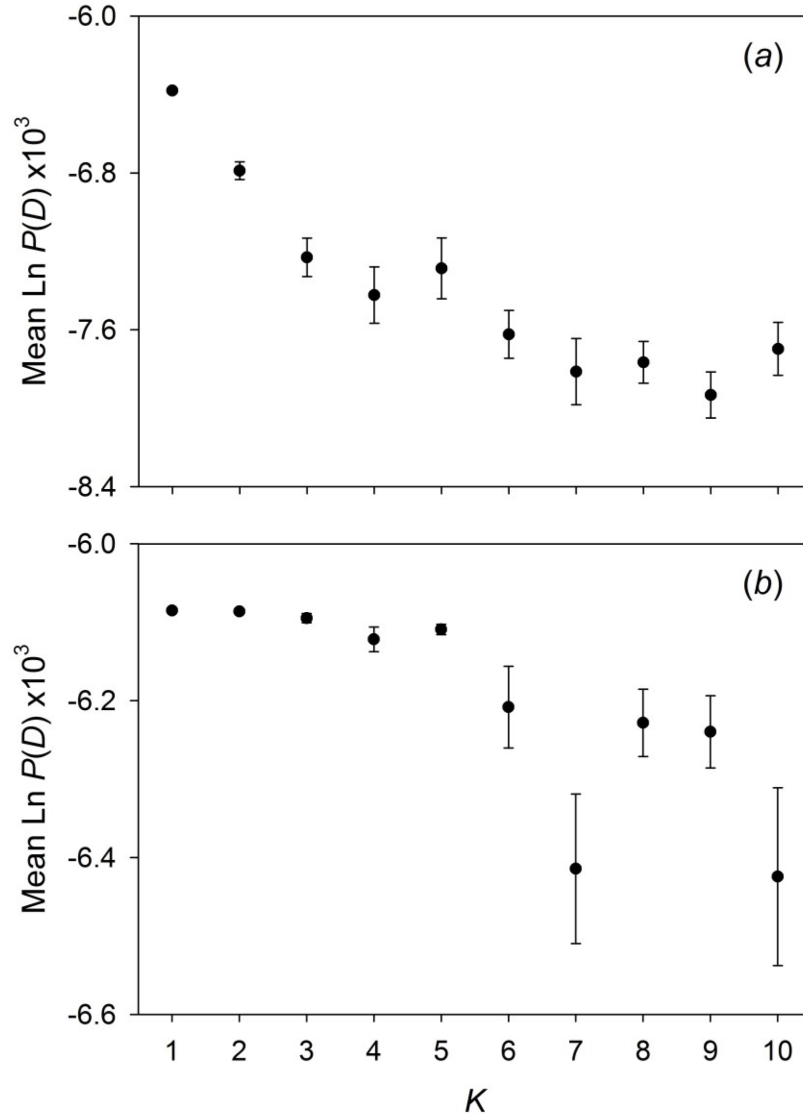
Supplementary Figure S3 Relationship between pairwise relatedness among mothers and the difference between individuals in their factor 1 scores. Mean and standard errors are shown for the data partitioned into roughly equal sized groups.

Supplementary Figure S4 Results of the BIO-ENV bootstrapping procedure. See Materials and methods for details. The y-axis shows the strength of correlation between genetic relatedness and pairwise Bray-Curtis similarity. On the x-axis, the chemicals are shown in decreasing order of importance, given by the number of subsamples in which the chemical was retained in the 'best' subset. Each chemical was progressively added to

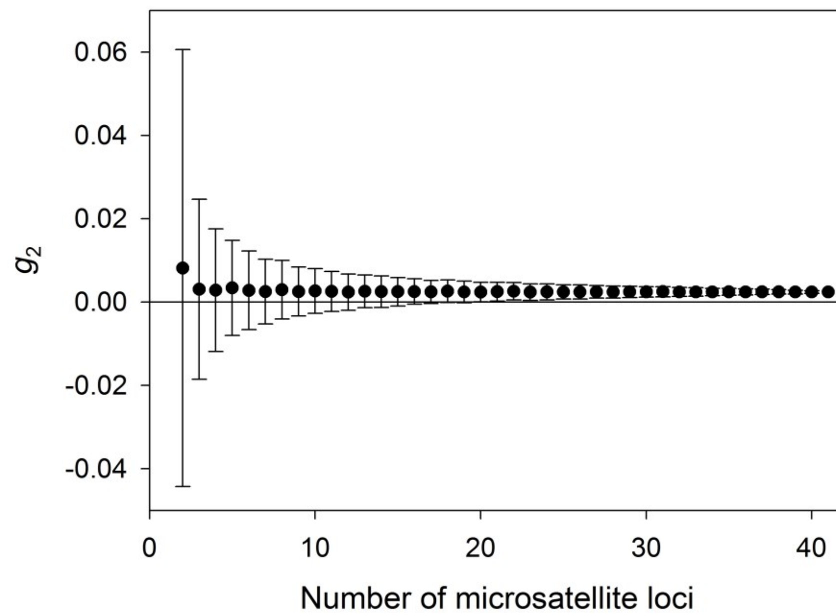
the calculation of Bray-Curtis similarity. The relationship between chemical similarity and relatedness is maximised for a subset of the ten most important chemicals.

Supplementary figures

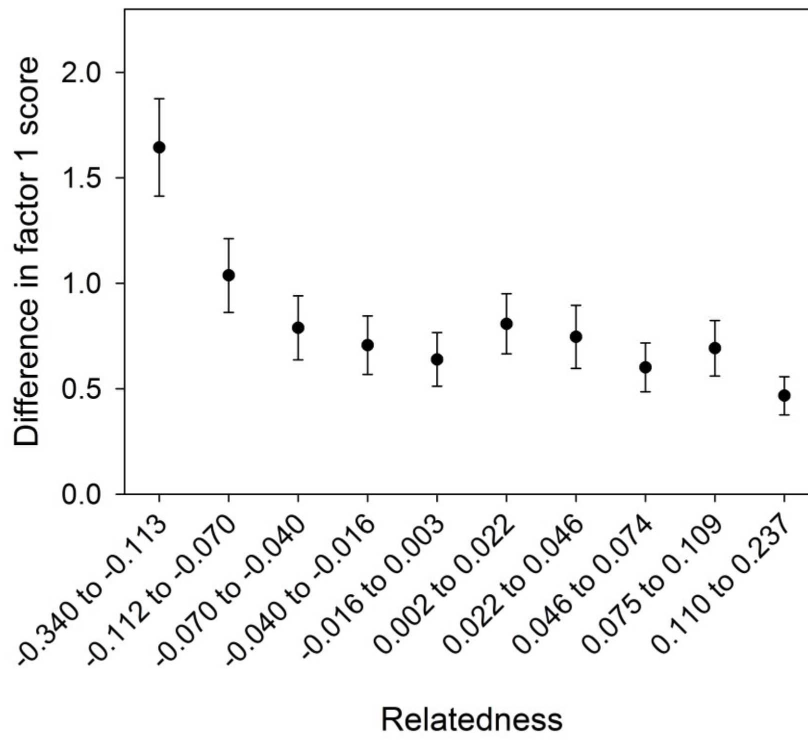
Supplementary Figure S1.



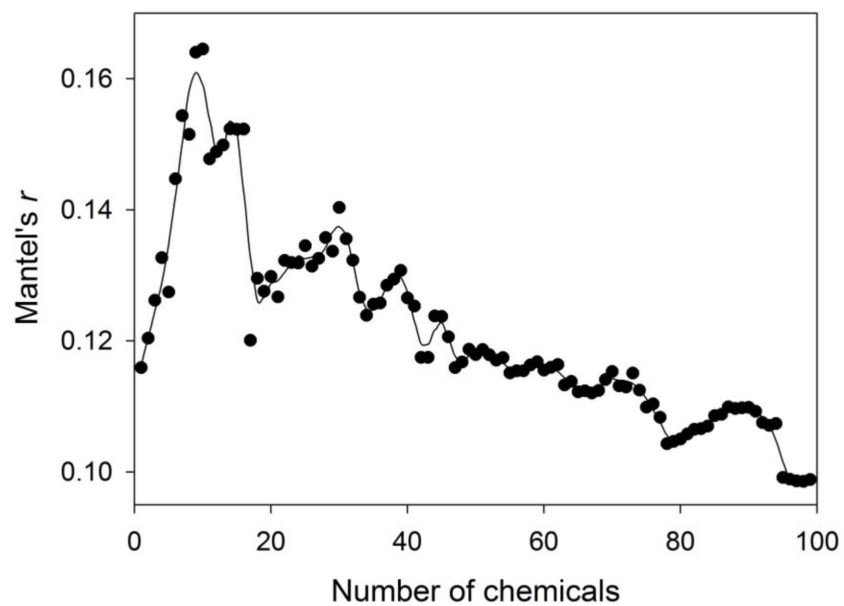
Supplementary Figure S2.



Supplementary Figure S3.



Supplementary Figure S4.



Supplementary tables

Supplementary table S1. Details of the 43 microsatellite loci used in this study together with their polymorphism characteristics in 41 mother-offspring pairs. Hardy-Weinberg equilibrium (HWE) *P*-values are shown separately for mothers and offspring, with significant values highlighted in bold. Values that remained significant following table-wide correction for the false discovery rate (FDR) are underlined. Loci Pv11 and ZcwCgDh5.16 were excluded from further analyses because they deviated significantly from HWE after FDR correction in either or both mothers or offspring. 'Mix' denotes the PCR mastermix into which each locus was multiplexed and 'T_a' denotes the annealing temperature used.

Locus	References	Mix	T_a	Number of alleles	Observed heterozygosity	HWE *P*-value	
						Mothers	Offspring
Pv9	Allen *et al.* [60]	1	53	10	0.691	**0.037**	0.129
Hg6.1	Allen *et al.* [60]	7	60	13	0.888	0.691	0.991
Hg6.3	Allen *et al.* [60]	1	53	12	0.901	0.942	0.074
Hg8.10	Allen *et al.* [60]	1	53	2	0.407	0.399	1.000
PvcA	Coltman *et al.* [61]	1	53	7	0.802	0.998	0.412
PvcE	Coltman *et al.* [61]	2	60	13	0.926	0.836	0.755
Aa4	Gemmell *et al.* [62]	4	60	6	0.720	0.685	0.419
Hg1.3	Gemmell *et al.* [62]	1	53	11	0.815	0.136	0.443
Pv11	Goodman [63]	8	60	11	0.329	0.399	<u>**0.000**</u>
OrrFCB2	Buchanan *et al.* [64]	2	60	11	0.888	0.394	0.234
OrrFCB7	Buchanan *et al.* [64]	2	60	10	0.813	0.391	0.649
M11a	Hoelzel *et al.* [65]	4	60	17	0.867	0.331	0.654
Lc28	Davis *et al.* [66]	4	60	9	0.875	0.988	0.542
Lw10	Davis *et al.* [66]	2	60	15	0.938	0.605	0.525
Zcc7t	Hernandez-Velazquez *et al.* [67]	7	60	13	0.896	0.568	0.492
ZcwCgDh1.8	Hernandez-Velazquez *et al.* [67]	3	60	9	0.744	0.608	**0.020**
ZcwDh3.6	Hernandez-Velazquez *et al.* [67]	4	60	4	0.234	0.590	0.392

ZcwCgDh4.7	Hernandez-Velazquez *et al.* [67]	3	60	13	0.924	0.842	0.336
ZcwCgDh5.16	Hernandez-Velazquez *et al.* [67]	2	60	7	0.500	**<u>0.000</u>**	**<u>0.000</u>**
ZcCgDh5.8	Hernandez-Velazquez *et al.* [67]	6	60	11	0.850	0.489	0.748
ZcwCgDh7tg	Hernandez-Velazquez *et al.* [67]	3	60	12	0.742	0.347	**0.026**
ZcwCgDhB.14	Hernandez-Velazquez *et al.* [67]	2	60	6	0.747	0.287	0.587
Zcwb09	Wolf *et al.* [68]	6	60	12	0.864	0.652	0.933
Zcwc03	Wolf *et al.* [68]	6	60	11	0.813	0.124	0.544
Zcwc11	Wolf *et al.* [68]	6	60	14	0.875	0.100	0.678
Zcwd02	Wolf *et al.* [68]	3	60	13	0.878	0.346	0.596
Zcwe03	Wolf *et al.* [68]	7	60	9	0.838	0.602	0.919
Ssl301	Huebinger *et al.* [69]	3	60	14	0.901	0.473	0.910
Zcwa05	Hoffman *et al.* [70]	5	60	14	0.896	0.645	0.999
Zcwb07	Hoffman *et al.* [70]	1	53	11	0.914	0.465	0.093
Zcwc01	Hoffman *et al.* [70]	2	60	11	0.823	0.345	0.511
Zcwe04	Hoffman *et al.* [70]	8	60	12	0.864	0.132	0.362
Zcwe12	Hoffman *et al.* [70]	8	60	8	0.768	0.595	0.881
Zcwf07	Hoffman *et al.* [70]	4	60	9	0.802	0.194	0.834
Ag1	Hoffman *et al.* [71]	3	60	10	0.813	0.706	0.733
Ag2	Hoffman *et al.* [71]	2	60	7	0.854	0.847	0.428
Ag3	Hoffman *et al.* [71]	2	60	2	0.420	0.454	0.311
Agaz2	Hoffman [72]	1	53	8	0.802	0.215	0.394
Agaz3	Hoffman [72]	2	60	5	0.629	0.896	0.288
Agaz5	Hoffman [72]	2	60	3	0.469	0.641	0.174
Agaz6	Hoffman [72]	2	60	4	0.765	0.750	0.156
Agaz10	Hoffman [72]	2	60	11	0.767	0.572	0.493
Zcwe05	unpublished	3	60	9	0.866	0.999	0.564

Supplementary table S2. Details of the mother-offspring pairs and their match probabilities calculated based on 41 microsatellite loci.

Colony	Mother ID	Offspring ID	Probability (%)
Special Study Beach	AGF11002	AGP11014	100
Special Study Beach	AGF11003	AGP11022	100
Special Study Beach	AGF11004	AGP11026	100
Special Study Beach	AGF11005	AGP11018	100
Special Study Beach	AGF11006	AGP11032	100
Special Study Beach	AGF11007	AGP11051	100
Special Study Beach	AGF11008	AGP11041	100
Special Study Beach	AGF11009	AGP11078	100
Special Study Beach	AGF11010	AGP11065	100
Special Study Beach	AGF11011	AGP11063	100
Special Study Beach	AGF11012	AGP11079	100
Special Study Beach	AGF11014	AGP11125	100
Special Study Beach	AGF11015	AGP11144	100
Special Study Beach	AGF11016	AGP11145	100
Special Study Beach	AGF11018	AGP11174	100
Special Study Beach	AGF11019	AGP11151	100
Special Study Beach	AGF11020	AGP11192	100
Special Study Beach	AGF11021	AGP11185	100
Special Study Beach	AGF11022	AGP11211	100
Special Study Beach	AGF11023	AGP11200	100
Freshwater beach	W8913mum	W8913pup	100
Freshwater beach	W8914mum	W8914pup	100
Freshwater beach	W8915mum	W8915pup	100
Freshwater beach	W8916mum	W8916pup	100
Freshwater beach	W8918mum	W8918pup	100
Freshwater beach	W8920mum	W8920pup	100
Freshwater beach	W8921mum	W8921pup	100
Freshwater beach	W8922mum	W8922pup	100
Freshwater beach	W8923mum	W8923pup	100
Freshwater beach	W8924mum	W8924pup	100
Freshwater beach	W8925mum	W8925pup	100
Freshwater beach	W8927mum	W8927pup	100
Freshwater beach	W8928mum	W8928pup	100
Freshwater beach	W8552/8258mum	W8552/8258pup	100
Freshwater beach	W8930mum	W8930pup	100
Freshwater beach	W8931mum	W8931pup	100
Freshwater beach	W8933mum	W8933pup	100
Freshwater beach	W8935mum	W8935pup	100
Freshwater beach	W8936mum	W8936pup	100
Freshwater beach	W8937mum	W8937pup	100
Freshwater beach	W8939mum	W8939pup	100

Supplementary table S3. Mean and standard deviation of pairwise Queller and Goodnight relatedness values.

	All individuals	Mothers	Offspring
Entire sample	0.009 +- 0.1	0.0008+-0.09	0.004+-0.09
Special study beach	0.016 +- 0.1	-0.005 +- 0.09	0.012 +- 0.09
Freshwater beach	0.011 +- 0.1	-0.004 +- 0.09	0.008 +- 0.10

Supplementary table S4. List of putative substances identified as being important for (a) chemical similarity within mother-offspring pairs; (b) chemical dissimilarity between the colonies; and (c) genetic relatedness. Substances are listed in decreasing order of importance, as measured by (a) the mean proportion of mother-pup similarity explained in the SIMPER analysis, (b) the percentage contribution towards dissimilarity between beaches, and (c) the number of occurrences within the best subsets identified by the BIOENV bootstrap procedure (see Methods for details). The chemical name and assignment probability are derived by a comparison of the empirical mass spectra with the most similar substance in the NIST library. The Kovats index was calculated for all substances with a retention time smaller than 28 min. For comparison, we provide the Kovats indices of the substances to which our compounds show the highest resemblance.

(a) Mother-offspring similarity					
Retention time (min)	Mean similarity explained (%)	Chemical name	Probability	Empirical Kovats Index	Kovats Index
19.723	15.54	Ethyl hexadecanoate (hexadecanoic acid ethyl ester)	58.3	1992	1993
15.458	12.25	1-Hexadecene	20	1591	1593
26.789	11.97	Squalene	46	2815	2790
16.397	11.30	8-Pentadecanone	94	1673	1648
19.525	10.87	Ethyl 9-hexadecenoate	87	1972	1977
21.405	8.49	Ethyl oleate	66	2175	2171
37.564	6.48	not identified	–	–	–
15.623	6.48	not identified	–	1606	–
33.637	6.28	Campesterol	71	–	–
30.804	6.03	Cholestanol	67	–	–
20.362	5.34	Heptadecanoic acid	69	2086	2067
17.409	4.79	not identified	–	1766	–

(b) Colony dissimilarity					
Retention time (min)	Similarity contribution (%)	Chemical name	Probability	Empirical Kovats Index	Kovats Index
15.458	3.01	1-Hexadecene	20	1591	1593
16.397	2.42	8-Pentadecanone	94	1673	1648
26.789	2.07	Squalene	46	2815	2790
19.525	1.97	Ethyl 9-hexadecenoate	87	1972	1977
21.405	1.89	Ethyl oleate	66	2175	2171
21.348	1.67	not identified	–	–	–
19.723	1.67	Ethyl hexadecanoate (hexadecanoic acid ethyl ester)	58.3	1992	1993
30.804	1.48	Cholestanol	67	–	–
38.518	1.44	not identified	–	–	–
17.409	1.33	not identified	–	1766	–

Retention time (min)	Occurrences in best subsets	Chemical name	Probability	Empirical Kovats Index	Kovats Index
20.511	1.29	not identified	–	–	–
33.637	1.27	Campesterol	71	–	–
21.575	1.21	Octadecanoic acid ethyl ester	85	2194	2194
15.742	1.18	not identified	–	–	–
19.665	1.13	not identified	–	–	–

(c) Relatedness

Retention time (min)	Occurrences in best subsets	Chemical name	Probability	Empirical Kovats Index	Kovats Index
36.941	315,926	not identified	–	–	–
19.525	250,140	Ethyl 9-hexadecenoate	87	1972	1977
13.124	245,569	not identified	–	–	–
20.362	214,830	Heptadecanoic acid	69	2086	2067
14.699	207,155	not identified	–	–	–
21.090	203,683	not identified	–	–	–
21.575	198,366	Octadecanoic acid ethyl ester	85	2194	2194
37.049	192,000	not identified	–	–	–
19.620	189,049	not identified	–	–	–
37.074	185,017	not identified	–	–	–

(*a*)

Mean Ln *P*(*D*) x10^3

-6.0
-6.8
-7.6
-8.4

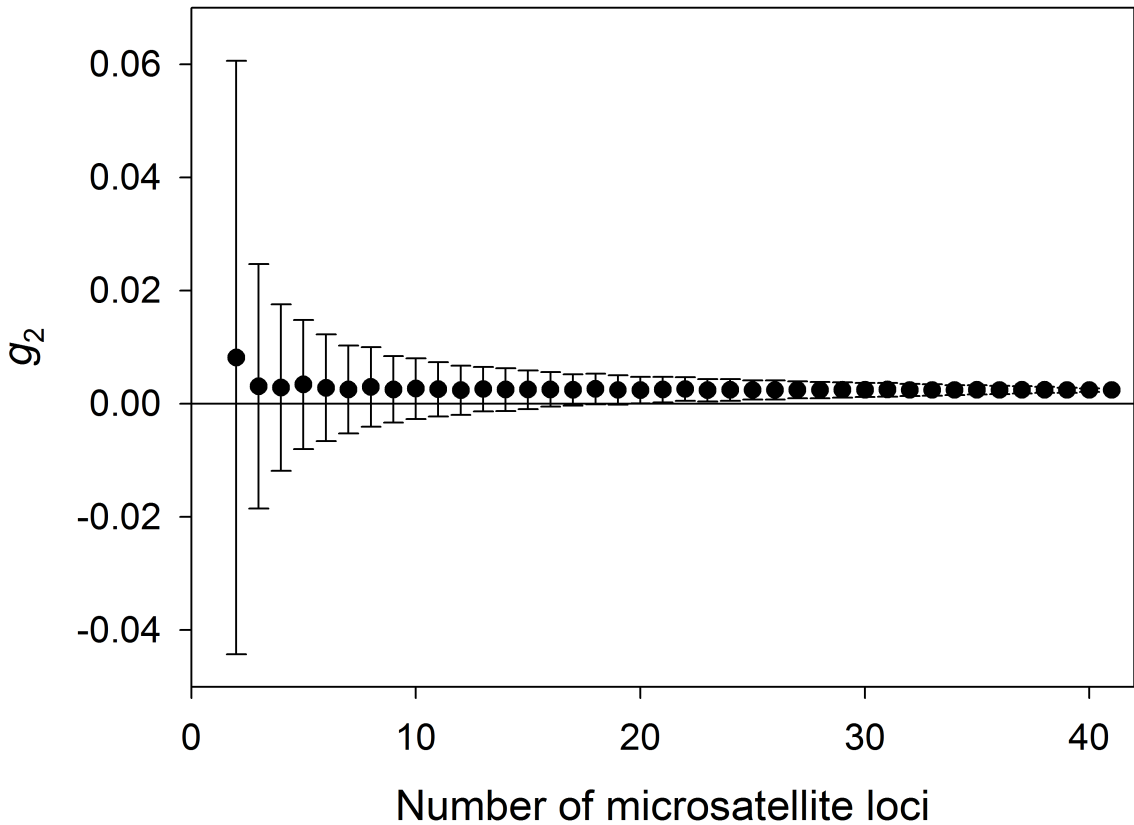
(*b*)

Mean Ln *P*(*D*) x10^3

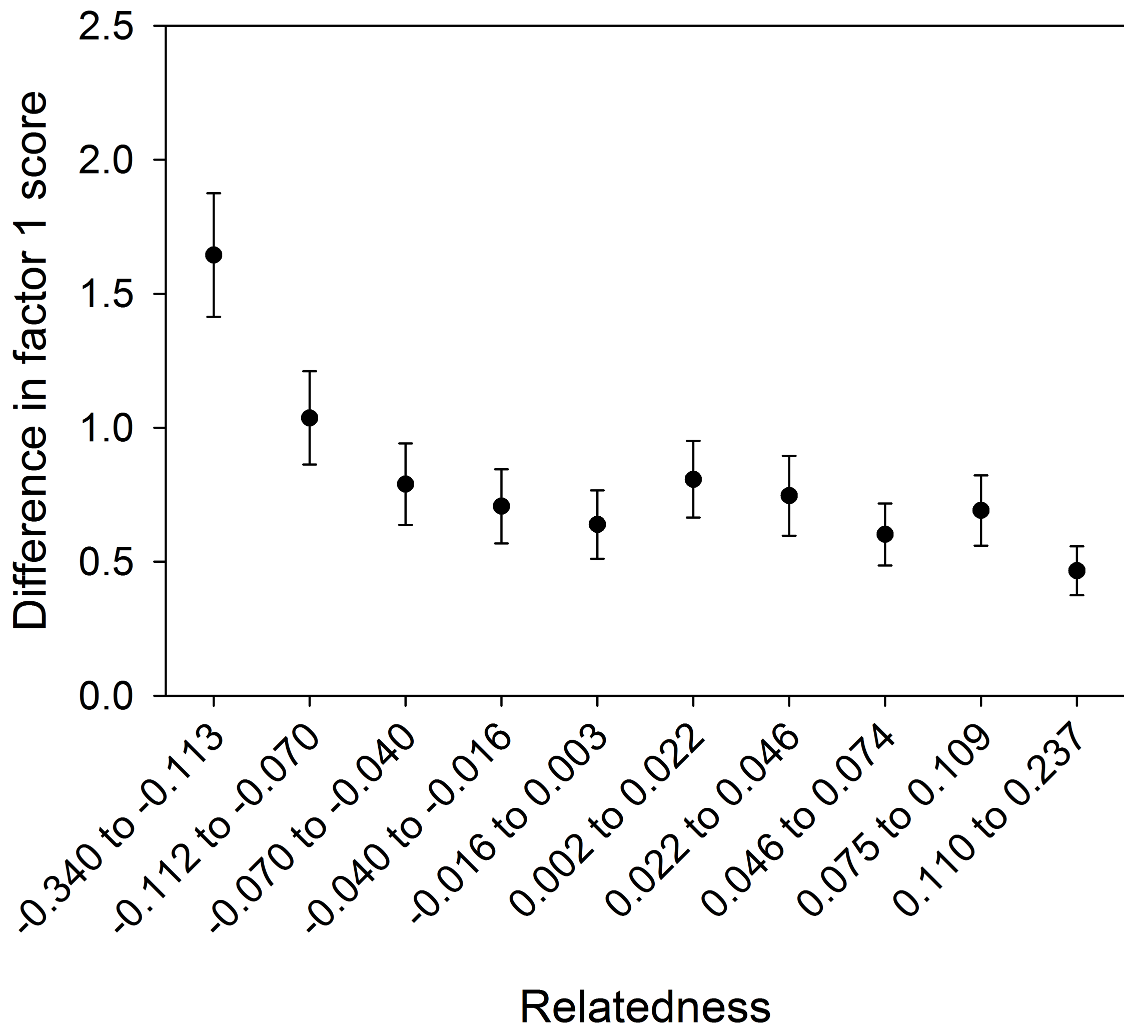
-6.0
-6.2
-6.4
-6.6

1 2 3 4 5 6 7 8 9 10

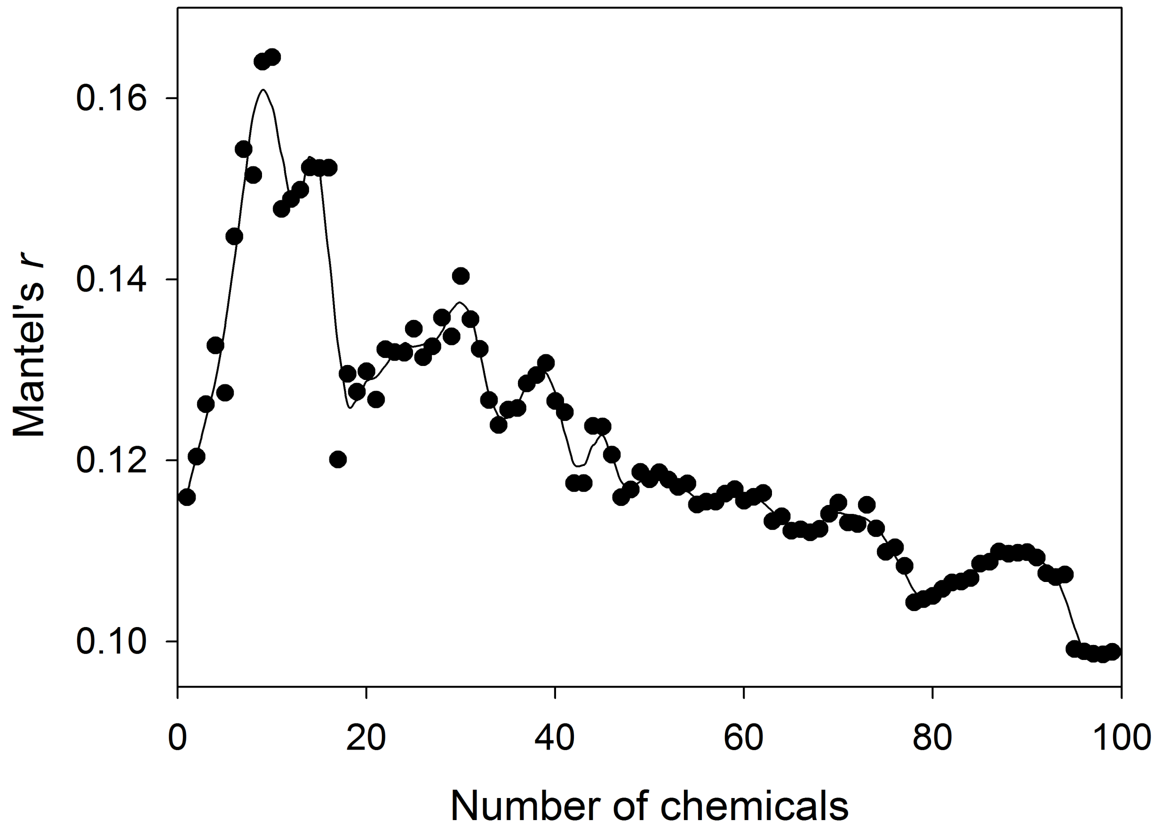
K



0.06
0.04
0.02
0.00
-0.02
-0.04
g_2
0
10
20
30
40
Number of microsatellite loci



Difference in factor 1 score
2.5
2.0
1.5
1.0
0.5
0.0
-0.340 to -0.113
-0.112 to -0.070
-0.070 to -0.040
-0.040 to -0.016
-0.016 to 0.003
0.002 to 0.022
0.022 to 0.046
0.046 to 0.074
0.075 to 0.109
0.110 to 0.237
Relatedness



Mantel's r
0.10
0.12
0.14
0.16
0
20
40
60
80
100
Number of chemicals