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How many genetic markers to tag an individual? An empirical assessment of false matching rates among close relatives

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Abstract. Genetic identification of individuals is now commonplace, enabling the application of tagging methods to elusive species or species that cannot be tagged by traditional methods. A key aspect is determining the number of loci required to ensure that different individuals have non-matching multi-locus genotypes. Closely related individuals are of particular concern because of elevated matching probabilities caused by their recent co-ancestry. This issue may be addressed by increasing the number of loci to a level where full siblings (the relatedness category with the highest matching probability) are expected to have non-matching multi-locus genotypes. However, increasing the number of loci to meet this "full-sib criterion" greatly increases the laboratory effort, which in turn may increase the genotyping error rate resulting in an upward-biased mark–recapture estimate of abundance as recaptures are missed due to genotyping errors. We assessed the contribution of false matches from close relatives among 425 maternally related humpback whales, each genotyped at 20 microsatellite loci. We observed a very low (0.5–4%) contribution to falsely matching samples from pairs of first-order relatives (i.e., parent and offspring or full siblings). The main contribution to falsely matching individuals from close relatives originated from second-order relatives (e.g., half siblings), which was estimated at ~9%. In our study, the total number of observed matches agreed well with expectations based upon the matching probability estimated for unrelated individuals, suggesting that the full-sib criterion is overly conservative, and would have required a 280% relative increase in effort. We suggest that, under most circumstances, the overall contribution to falsely matching samples from close relatives is likely to be low, and hence applying the full-sib criterion is unnecessary. In those cases where close relatives may present a significant issue, such as unrepresentative sampling, we propose three different genotyping strategies requiring only a modest increase in effort, which will greatly reduce the number of false matches due to the presence of related individuals.

Key words: *abundance estimation; conservation; error rates; genetic tagging; humpback whale; mark–recapture;* Megaptera novaeangliae; *microsatellite; monitoring; number of loci; probability of identity; relatedness.*

Introduction

Although it was introduced only a decade ago, genetic tagging of individuals has now become a widely adopted approach in ecology and conservation (Waits and Paetkau 2005, Schwartz et al. 2007). Genetic tagging has been employed primarily to estimate abundance by mark–recapture methods of natural animal populations, such as humpback whales (e.g., Palsbøll et al. 1997*a*) and grizzly bears (e.g., Woods et al. 1999, Kendall et al. 2009). Some more imaginative applications include estimating the number and duration of dead whales in the market place (Baker et al. 2007), and abundance estimation of breeding males from identification of paternal genotypes in broods of offspring (Pearse et al. 2001). Although genetic tagging is equivalent to traditional tagging methods in many aspects, the methodology has its own unique features owing to its hereditary basis as well as the experimental procedures involved (Bonin et al. 2004, Pompanon et al. 2005, Waits and Paetkau 2005). The identification of individuals from their multi-locus genotype must account for two kinds of possible errors: incorrectly inferring that (1) samples with different genotypes originate from different individuals, and (2) samples with identical genotypes originate from the same individual. The former source of error (1) is caused by experimental errors, in the field or during the

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genotyping of samples, and has been the focus of multiple studies and reviews (Taberlet et al. 1999, Bonin et al. 2004, Pompanon et al. 2005, Waits and Paetkau 2005). The latter kind of error (2) is typically, but not always, a result of genotyping too few loci, which in turn causes different individuals to have identical multi-locus genotypes by chance (Woods et al. 1999, Waits et al. 2001, Paetkau 2004). This aspect is usually dealt with in terms of I (the probability of identity), which denotes the probability that two different individuals have identical multi-locus genotypes (Paetkau et al. 1995) when a finite number of loci are genotyped. The sampling probabilities of alleles used in the estimation of I are usually the observed allele frequencies (Paetkau and Strobeck 1994) and may be conditioned upon the level of relatedness among individuals (Woods et al. 1999, Waits et al. 2001). Most studies that initially employed genetic tagging estimated I assuming that all individuals were unrelated, and many still do. However, Waits et al. (2001) undertook an assessment of three empirical data sets and found that the observed number of samples that matched at four, six, or eight loci was much higher (in some cases threefold) than that expected from the estimate of I for unrelated individuals. The authors suggested that the discrepancy between the observed and expected number of matching genotypes stemmed from the inclusion of related individuals, because I is higher for related individuals than for unrelated individuals. Consequently, the authors proposed (as did Woods et al. 1999), to use I for full siblings, instead of I for unrelated individuals, to guide the decision of how many loci to genotype for the purpose of genetic tagging. This full-sibling criterion (henceforth termed the FS criterion) makes sense in theory, but presents a number of nontrivial practical challenges and potential biases (McKelvey and Schwartz 2004, Paetkau 2004), which we will briefly review.

A large number of studies employing genetic tagging use noninvasively collected samples, which contain DNA of suboptimal quality (Taberlet et al. 1999, Valiere and Taberlet 2000, Pompanon et al. 2005). With such samples, each additional locus to be genotyped represents a substantial increase in effort (Taberlet et al. 1996, 1999, Paetkau 2004). Second, the overall multi-locus error rate is positively correlated with the number of loci genotyped (assuming that the per-locus error rate is constant) (Bonin et al. 2004). Accordingly, increasing the number of loci will potentially increase the number of samples from the same individual that appear to have different multi-locus genotypes, i.e., some matching samples will be missed. In turn, missing such matches results in erroneous estimates of the parameter values typically estimated from genetic tagging, such as abundance (Mills et al. 2000, Roon et al. 2005, Waits and Paetkau 2005, Schwartz et al. 2007). Missed identifications will lead to an overestimation of abundance (Mills et al. 2000, Waits and Leberg 2000, Creel et al. 2003, Paetkau 2003, Roon et al. 2005; but see Wright et al. 2009), which in turn would lead to a conclusion of decreasing endangerment, and consequently (if incorrect) potentially could increase the risk of extinction if an increased abundance estimate results in a reduction of conservation efforts.

The conclusion arrived at by Waits et al. (2001), that pairs of related individuals were the cause of the much higher than expected numbers of matching genotypes, has only been subject to very limited assessment (e.g., Paetkau 2003). Although related individuals (particularly first-order relatives, i.e., parent–offspring and full-sibling dyads) have much higher I values compared to unrelated individuals, a random population sample will usually contain few dyads of closely related individuals. Accordingly, even though I for full siblings is several-fold higher than I for unrelated individuals, the number of full-sibling dyads contained in a random sample of individuals is expected to be orders of magnitude fewer than the number of dyads of unrelated individuals (e.g., Paetkau 2003). Consequently, the contribution from dyads of close relatives to the total number of incorrectly matching samples (i.e., samples from different individuals with identical multi-locus genotypes) is in fact expected to be low. If, on the other hand, a sample contains a large proportion of closely related individuals, this will in turn increase the allele frequencies of those alleles shared among such close relatives and thus also I for unrelated individuals.

A closer inspection of the data presented by Waits and coworkers (Table 1 in Waits et al. 2001) revealed that in those cases where the discrepancy between the expected and observed I were largest, the observed number of matches was usually low (from one to four matches; see Table 1) except for two cases (Montana and Banff) of gray wolves (*Canis lupus*). However, when compared to an expectation of zero matches, such differences became very large in relative terms. Last, but not least, due to the nature of sample collection in some of these studies, and the inability in some cases to ground-truth genetic identifications with other means of individual identification, it may be that some of the observed matches included in these data sets may have originated from the same individual, rather than closely related individuals.

In this study, we conducted an empirical assessment of the effect of closely related individuals upon matching rates. The very few earlier assessments of the effect of close relatives upon matching probabilities were based upon relationships inferred from a few known mother–offspring pairs such as in Paetkau et al. (2003). Our study was based upon a data set composed of genotypes at 20 microsatellite loci genotyped in each of 425 individual humpback whales (*Megaptera novaeangliae*), which summer in the Gulf of Maine at the eastern North American sea border. In total, ~900 humpback whales reside in the Gulf of Maine from spring through the fall (Clapham et al. 2003). During the late fall, most humpback whales migrate to the West Indies to mate

TABLE 1. Number of expected matching samples (estimated from *I*, the probability of identity) and observed matching samples in Waits et al. (2001).

Species and location	Samples	Loci	Expected		Observed	
			$I_{[UR]}$	No. matches	Matching probability	No. matches
Brown bear						
Scandinavia, South	157	4	1.3×10^{-3}	16	3.1×10^{-3}	38
		6	1.0×10^{-4}	1	7.3×10^{-4}	9
		8	1.7×10^{-6}	0	1.6×10^{-4}	2
Scandinavia, Central	86	4	1.4×10^{-3}	5	2.2×10^{-3}	8
		6	1.3×10^{-4}	0	5.5×10^{-4}	2
		8	1.3×10^{-6}	0	2.7×10^{-4}	1
Scandinavia, North–South	107	4	3.9×10^{-4}	2	1.4×10^{-3}	8
		6	7.1×10^{-6}	0	7.1×10^{-4}	4
		8	1.9×10^{-7}	0	1.8×10^{-4}	1
Wolf						
Montana, USA	64	4	3.6×10^{-3}	7	5.5×10^{-2}	111
		6	1.9×10^{-4}	0	2.5×10^{-2}	50
		8	2.5×10^{-5}	0	8.2×10^{-3}	17
Fort Saint Johns, Canada	41	4	2.2×10^{-3}	2	3.7×10^{-2}	30
		6	9.3×10^{-5}	0	3.7×10^{-3}	3
		8	1.7×10^{-7}	0	1.2×10^{-3}	1
Hinton, Canada	33	4	3.3×10^{-3}	2	1.5×10^{-2}	8
		6	3.3×10^{-4}	0	7.6×10^{-3}	4
		8	3.7×10^{-5}	0	N/A	0
Banff, Canada	32	4	2.8×10^{-3}	1	3.0×10^{-2}	15
		6	3.7×10^{-4}	0	2.0×10^{-2}	10
		8	1.6×10^{-5}	0	2.0×10^{-2}	10
Wombat						
Epping Forest, Australia	28	4	2.3×10^{-3}	1	1.4×10^{-2}	5
		6	4.3×10^{-4}	0	5.1×10^{-3}	2
		8	1.7×10^{-4}	0	1.1×10^{-3}	0

Notes: *I* denotes the probability that two different individuals have identical multi-locus genotypes (Paetkau et al. 1995). Data are reconstructed from Waits et al. (2001); brown bear data for Sweden are from Swenson et al. (1994). The number of expected and observed matches was calculated by multiplying the number of pairwise comparisons with *I* (in case of the expected matches) and the observed matching probability (in case of the observed matches).

and calve during the winter (Katona and Beard 1991). Longitudinal studies of individually identified humpback whales in the North Atlantic have shown that calves born in the West Indies are subsequently recruited to their mother's summer feeding area during their first year (Martin et al. 1984, Clapham and Mayo 1987), and that each individual consistently returns to its maternal summer feeding area every spring for the remainder of its life. This maternally driven site fidelity to the Gulf of Maine summer feeding area implies that our sample from the Gulf of Maine population contains many dyads of maternal relatives; thus our sample of individuals is expected to exhibit elevated rates of falsely matching genotypes, as suggested by Waits et al. (2001).

Our assessment revealed that the greatest contribution to erroneous matches from dyads of close relatives does not originate from first-, but rather from second-order relatives. The proportion of dyads of first-order relatives among our samples was very low, resulting in a minor overall effect on the false matching rates. In conclusion, our study suggests (as pointed out earlier by Paetkau 2003) that the FS criterion probably is overly stringent. For cases in which close relatives might present an issue (e.g., due to an unrepresentative sampling design), we propose three laboratory strategies by which the level of genotyping may be greatly reduced relative to the FS criterion, while still maintaining a high degree of statistical rigor.

MATERIALS AND METHODS

Sample collection and DNA extraction

We obtained skin biopsies or sloughed skin from free-ranging humpback whales and their documented offspring (see Plate 1). Humpback whales can be uniquely identified by the pigmentation pattern on the ventral side of their tail, the pattern of serrations on the trailing edge of the tail, and the shape of the dorsal fin (Katona and Whitehead 1981, Katona and Beard 1991). The majority of samples were obtained in the Gulf of Maine during the period 1990–1999. A few individuals that are known to summer in the Gulf of Maine were sampled in the West Indies in 1990, 1991, or 1992. Mother and offspring relationships among samples were based on identification data and behavioral observations made during the one-year period of offspring dependency. Total-cell DNA was extracted from skin biopsies by standard phenol : chloroform extraction and the DNA was resuspended in 1xTE (67 mmol/L Tric-HCl, pH 8.8) (Sambrook and Russell 2001).

TABLE 2. Identity by descent (IBD) coefficients (k_{0-2}) for each relationship category.

Relationship category	k_0	k_1	k_2
Parent and offspring	0.00	1.00	0.00
Full siblings	0.25	0.50	0.25
Second-order relatives	0.50	0.50	0.00
Unrelated individuals	1.00	0.00	0.00

Genotyping

The genotype was determined at the following microsatellite loci: EV001, EV037, EV094, and EV096 (all dinucleotide loci; Valsecchi and Amos 1996), TAA031, GATA053, GATA098, GATA417, GGAA520 (letters denote repeat motif; Palsbøll et al. 1997*b*), GT011 (letters denote repeat motif, Bérubé et al. 1998), GT015, GT023, GT101, GT195, GT211, GT271, GT307, GT575 (letters denote repeat motif; Bérubé et al. 2000), and RW004-10 (also a dinucleotide locus; Waldick et al. 1999). For each locus one oligo-nucleotide primer was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. PCR amplifications were conducted in 10-μL volumes, each with 10 ng of genomic DNA, 67 mmol/L Tris-HCl, pH 8.8, 2 mmol/L $MgCl_2$, 16.6 mmol/L $(NH_4)_2SO_4$, 10 mmol/L β-mercaptoethanol, 0.2 mmol/L dNTPs, 1 mmol/L of the unlabeled oligo-nucleotide primer, 40 μmol/L of the end-labeled oligo-nucleotide primer, as well as 0.4 units of *Taq* DNA polymerase under the published conditions (Valsecchi and Amos 1996, Palsbøll et al. 1997*b*, Bérubé et al. 1998, 2000, Waldick et al. 1999). The amplification products were separated by electrophoresis through a denaturing 5% polyacrylamide gel. After electrophoresis, the gel was fixed in 5% ethanol and 5% acetic acid for 40 min, followed by a 15-min rinse in tap water. The fixed polyacrylamide gel was dried at 80°C for 45 min and autoradiography performed with Kodak BioMax film for 5–48 h depending on the intensity of radioactive signal. The size of the amplification products was estimated by comparison to [γ-^{32}P]ATP-labeled λM13 sequences. Subsequent nonradioactive sizing of PCR products was conducted under similar electrophoresis conditions using an ABI Prism 377 where one oligo-nucleotide primer was fluorescently labeled (and added in 1 mmol/L concentration). The size-standard TAMRA-500 (Applied Biosystems Inc., Foster City, California, USA) was added to each lane for allele sizing. Alleles were scored using GenoTyper (ver. 3.7, Applied Biosystems Inc.) and checked manually. All amplification experiments and subsequent electrophoresis included a minimum of six positive controls (i.e., individuals of known genotype) and one negative control per 48 genotyping PCRs.

Data analysis

Known mother–calf pairs and re-identifications of known individuals (identified from their natural markings) were used to assess genotyping error rates.

We use the following terminology in this study to denote I for different types of relationships: $I_{[UR]}$, denotes I for a dyad of unrelated individuals. $I_{[SIBS]}$ denotes I for a dyad of full siblings, whereas $I_{[PO]}$ denotes I for a parent and offspring dyad. $I_{[2ND]}$, refers to I for second-order relationships, such as half siblings, grandparent and grandchild, or avuncular. Hence, our $I_{[SIBS]}$ is the same as $P_{(ID)sib}$ (Waits et al. 2001) and $I_{[UR]}$ equals I (Paetkau et al. 1995) or $P_{(ID)}$ (Waits et al. 2001).

The probability of identical genotypes in a dyad is a function of the probability of identity by descent (IBD), which relates to IBD coefficients (Blouin 2003). Let k_0, k_1, and k_2 denote the probability that two individuals share zero, one, or two alleles by descent. The values for k_0, k_1, and k_2 differ among relationship categories. However, the probability of identical genotypes in two different individuals (denoted $\Pr[I \mid k_i]$, where i takes the values of 0, 1, or 2) is the same across all relationship categories for k_0, k_1, and k_2, respectively. Accordingly, I for a dyad of a specific relationship category at a single locus with N_a alleles (where p_i and q_j denotes the frequency of the ith and jth allele) becomes

$$I = \sum_{i=0}^{2} k_i \times \Pr[I \mid k_i] \tag{1}$$

where

$$\Pr[I \mid k_0] = \sum_{i=1}^{K} p_i^4 + \sum_{i=0, j>i}^{N_a} (2p_i q_i)^2 \tag{2}$$

$$\Pr[I \mid k_1] = \sum_{i=1}^{N_a} p_i^2 \tag{3}$$

and

$$\Pr[I \mid k_2] = 1. \tag{4}$$

The IBD coefficients for different relationship categories are listed in Table 2.

The number of expected and observed matches for each kind of $I_{[UR]}$, $I_{[2ND]}$, $I_{[SIBS]}$, and $PI_{[PO]}$ was estimated for the 425 samples for each locus separately as well as for four nonoverlapping sets of loci. These four sets of loci were decided upon by adding loci (starting with the locus with the lowest $I_{[UR]}$) as long as at least one pair among the 425 individuals had identical multi-locus genotypes.

All possible dyads of individuals were assigned to a relatedness category that was estimated from all 20 microsatellite genotypes using MLRelate (ver. 22 April 2008; Kalinowski et al. 2006). MLRelate uses LOD (log of the odds) scores (Thompson 1975) to infer the most likely relationship category for a dyad of individuals.

For each set of loci (and subsets of each set), the observed number of dyads with matching multi-locus genotypes per relatedness category (estimated from the complete 20-locus genotypes) was counted. The expected number of matches ($E(m)$) was estimated as

$$E(m) = n \times (n-1)/2 \times \hat{I}_R \tag{5}$$

where n denotes the number of genotyped samples and $\hat{I}_R$ is the estimate of I for the relatedness category R.

The probability of non-matching samples (Pr[$-m$]) from the same individual due to a genotyping error rate r (same for all loci) was estimated as

$$\Pr[-m] = 1 - (1 - r)^{N_{\text{loci}}} \quad (6)$$

where N_{loci} denotes the number of loci genotyped.

Results

In total, 516 different samples were genotyped at six loci (GATA028, GATA053, GATA098, GATA417, GGAA520, and TAA031). Among these 516 samples were 93 known duplicate samples (i.e., individuals identified by natural markings). In all 93 cases but two, the six locus genotypes agreed 100%. In the two discrepant cases, the genotypes differed at five or six loci, respectively. These samples were inferred as representing mislabeled samples (either in the field or during DNA extraction). Because the mislabeling has no effect upon the estimations conducted in this study, these two samples were included in our analysis. After removal of the 91 duplicate samples, the remaining 425 samples (each representing an individual humpback whale) were genotyped at an additional 14 microsatellite loci (see *Materials and methods*).

Among the 425 individuals were 96 known mother and calf dyads. All of these 96 dyads matched (as expected assuming Mendelian segregation) at a minimum of one allele at each of the 20 microsatellite loci. All of these mother and calf dyads also had identical mitochondrial control region DNA sequences (data not shown), as expected given the maternal inheritance of the mitochondria. Accordingly, in all cases that we were able to check for genotyping errors, none were detected.

Of the total 8500 possible genotypes in these 425 individuals, we were unable to collect unambiguous genotypes in 65 instances, resulting in an overall scoring rate of 99.2%. No single individual was lacking data at more than three loci.

Using MLRelate (Kalinowski et al. 2006) and all successfully typed genotypes from the 20 microsatellite loci in each individual, 271 dyads were inferred as first-order relatives (154 parent–offspring and 117 full sibling dyads), 8491 dyads as second-order relatives, and the remaining 81 338 pairs as unrelated. It should be noted that with data from 20 microsatellite loci, it was in most cases impossible to discriminate between different relatedness categories with a reasonable degree of statistical confidence (i.e. at the 5% level). As expected, I increased with the degree of relatedness; the more closely related, the higher the estimated value of I.

The trend, in terms of the number of matching genotypes per dyad, within each inferred relatedness category, was as expected; the dyads inferred as full siblings had the highest matching rates, followed by dyads inferred as parent and offspring, second-order relatives, and unrelated, respectively (Tables 3 and 4). The fraction of matching genotypes stemming from each relatedness category was, for single loci, on average, 1% (range 0.5–1.9%) from dyads inferred as first-order relatives, 15% (range 6.6–19.5%) from dyads inferred as second-order relatives, and 84% (range 79–92.6%) from dyads inferred as unrelated individuals (Table 3). For two loci (Table 4), the picture changed slightly and, on average, 4% and 25% of the matches originated from dyads inferred as first- and second-order relationships, whereas dyads inferred as unrelated individuals accounted for an average of 71% of all matches.

Looking at the four multi-locus sets next, matching dyads of inferred first-order relatives disappeared as more loci were added to a set (Table 4). At the maximum number of loci, matches (ranging between one and four matches) were between dyads inferred as second-order relatives and dyads inferred as unrelated individuals, in equal proportions (Table 4).

Comparing the observed and expected number of matches (calculated using $I_{[\text{UR}]}$) at single loci yielded a ratio of observed to expected at 0.99 (range 0.89–1.07; see Table 5).

We estimated that a total of 14 loci (starting with the locus with the lowest I) would be needed to satisfy the FS (full-sibling) criterion (i.e., using $I_{[\text{SIBS}]}$ as I) for a target of fewer than 0.1 expected random matches. In contrast, to meet the same expectation using $I_{[\text{UR}]}$ or $I_{[\text{2ND}]}$ as the basis for the estimation, an estimated five or eight loci, respectively, would be needed.

Discussion

Our study aimed to complement and further assess the findings by Waits et al. (2001), namely that the presence of close relatives results in a substantial increase in the total number of erroneously matching samples. This finding has severe ramifications upon the application of genetic tagging for genetic monitoring (Schwartz et al. 2007). Because the relative proportions of different relatedness categories contained in a random population sample are rarely known, this observation has posed a challenge in terms of planning the effort (i.e., the number of loci to genotype) required for a robust implementation of genetic tagging (McKelvey and Schwartz 2004, Paetkau 2004). As pointed out by Paetkau (2004), increasing the number of loci to satisfy the most rigorous criterion (i.e., the FS criterion) is nontrivial, especially if a study is based upon noninvasively obtained DNA (Taberlet et al. 1996, 1999). In our case, applying the FS criterion would result in an increase in the number of loci from five to 14, representing a 280% increase in effort. Apart from the substantial increase in labor and costs, what is the effect of increasing the number of loci? An increase in the total number of loci per genotype will result in an increase in the overall per-genotype error rate as well (Paetkau 2003, 2004, McKelvey and Schwartz 2004). For instance, if the per-

TABLE 3. Single-locus estimates of variation, probability of identity *I*, and observed numbers of matches for each inferred relatedness category.

			Parent and offspring			Full siblings		
Locus	N_a	H_e	$I_{[PO]}$	Matches/pairs	Fraction of all matches	$I_{[SIBS]}$	Matches/pairs	Fraction of all matches
GATA417	15	0.88	0.1227	13/154	0.005	0.3181	25/117	0.010
EV037	14	0.87	0.1321	17/152	0.007	0.3239	31/114	0.012
GATA053	9	0.83	0.1659	22/153	0.006	0.3449	25/117	0.006
RW004-10	14	0.83	0.174	22/152	0.005	0.35	28/114	0.006
GT011	8	0.83	0.1742	21/154	0.004	0.3503	22/117	0.005
GGAA520	27	0.81	0.1969	28/148	0.006	0.3621	45/107	0.010
EV096	9	0.82	0.1796	25/153	0.005	0.3536	33/116	0.007
TAA031	13	0.82	0.1821	42/150	0.005	0.3552	33/115	0.007
GT211	7	0.82	0.1811	26/154	0.005	0.3551	27/117	0.005
GT023	8	0.8	0.1944	31/154	0.005	0.3631	44/117	0.008
GT015	14	0.81	0.2042	27/150	0.005	0.3689	36/108	0.006
GT101	9	0.76	0.247	29/152	0.003	0.3973	42/116	0.005
GT575	11	0.72	0.2801	30/154	0.003	0.4149	50/117	0.006
GATA098	8	0.67	0.3347	43/153	0.004	0.452	38/117	0.003
GT307	7	0.68	0.33	45/154	0.004	0.4503	50/117	0.004
EV094	5	0.68	0.3114	33/154	0.002	0.4438	62/117	0.005
GT195	5	0.65	0.3462	59/154	0.003	0.4707	55/117	0.003
GT271	10	0.59	0.4165	55/154	0.003	0.5092	47/117	0.002
EV001	4	0.6	0.4014	46/154	0.002	0.5048	55/117	0.003
GATA028	10	0.49	0.5304	73/151	0.003	0.5898	52/109	0.002
Average fraction of all matches					0.004			0.006

Notes: N_a is the number of alleles detected; H_e is the estimate of expected heterozygosity; *I*, the probability that two different individuals have identical multi-locus genotypes, is estimated for the specific relationship category (see *Materials and methods* for definitions). Matches/pairs is the observed number of matching dyads of the inferred relatedness category and the total number of dyads of the inferred relatedness category among all genotyped individuals at the target locus. Also shown is the fraction of all matches observed at the target locus from the specific relatedness category.

TABLE 4. Observed number of matches by inferred relatedness category for sets of microsatellite loci.

			Parent and offspring			Full siblings		
			Matches/pairs			Matches/pairs		
Category	Loci	Total matches/ samples	No.	Fraction	Fraction of total matches	No.	Fraction	Fraction of total matches
Set 01								
MM-2	GATA417 + EV037	77/421	1/152	0.007	0.013	3/114	0.026	0.039
MM-1	+ GATA053	3/399	0/128	0	0	0/96	0	0
Set 02								
MM-4	RW004-10 + GT011	225/420	2/152	0.013	0.009	5/114	0.044	0.022
MM-3	+ GGAA520	9/411	0/146	0	0	0/105	0	0
MM-2	+ EV096	3/409	0/145	0	0	0/104	0	0
MM-1	+ TAA031	1/407	0/141	0	0	0/102	0	0
Set 03								
MM-3	GT211 + GT023	358/423	11/154	0.071	0.031	7/117	0.060	0.020
MM-2	+ GT015	31/414	0/150	0	0	2/108	0.019	0.065
MM-1	+ GT101	4/411	0/148	0	0	0/107	0	0
Set 04								
MM-5	GT575 + GATA098	1179/424	8/153	0.052	0.007	17/117	0.145	0.014
MM-4	+ GT307	146/424	2/153	0.013	0.014	9/117	0.077	0.062
MM-3	+ EV094	20/424	0/153	0	0	3/117	0.026	0.150
MM-2	+ GT195	5/424	0/153	0	0	2/117	0.017	0.400
MM-1	+ GT271	1/424	0/153	0	0	0/117	0	0

Notes: Category follows the definition by Paetkau (2003), where MM-1 and MM-2 denote the full complement of loci minus 1 or minus 2, respectively. The full complement of loci is the set of loci assumed sufficient to identify individuals. In column 2, a "+" denotes the locus listed plus those above from within the same set. Column 3 gives the total number of observed matching individuals and the total number of samples with complete genotypes at all loci in the set. For each relatedness category, numbers of matches/pairs are given, along with the proportion of matches (e.g., for set 01, MM-2, parent and offspring, $1/152 = 0.007$; $1/77 = 0.013$).

TABLE 3. Extended.

Second-order relatives			Unrelated individuals		
$I_{[2ND]}$	Matches/pairs	Fraction of all matches	$I_{[UR]}$	Matches/pairs	Fraction of all matches
0.0748	503/8425	0.195	0.027	2038/80 557	0.790
0.0818	489/8409	0.189	0.0315	2048/80 578	0.792
0.1068	628/8448	0.163	0.0477	3179/80 958	0.825
0.1131	797/8340	0.183	0.0521	3517/79 804	0.806
0.1135	743/8454	0.158	0.0529	3912/80 951	0.833
0.1257	817/8101	0.175	0.0546	3780/77 964	0.809
0.1175	783/8337	0.165	0.0555	3912/79 804	0.823
0.1193	743/8354	0.150	0.0564	4140/80 212	0.838
0.1196	837/8453	0.169	0.058	4063/80 952	0.820
0.129	913/8448	0.161	0.0636	4699/80 957	0.826
0.1357	898/8118	0.150	0.0672	5019/77 944	0.839
0.171	1234/8359	0.144	0.095	7285/80 204	0.848
0.1898	1407/8491	0.158	0.0996	7408/81 338	0.833
0.2366	1766/8448	0.143	0.1384	10 483/80 958	0.850
0.2357	1760/8491	0.066	0.1414	10 701/81 338	0.926
0.2319	1790/8491	0.134	0.1524	11 486/81 338	0.859
0.2682	2129/8491	0.121	0.1903	15 391/81 338	0.873
0.3102	2399/8491	0.127	0.2039	16 377/81 338	0.868
0.3089	2371/8491	0.123	0.2165	16 873/81 338	0.872
0.4144	3119/8369	0.122	0.2985	22 405/80 202	0.874
		0.150			0.840

locus error rate is 1% (which appears to be a common level of error rate; Bonin et al. 2004), increasing the number of loci from five to 14 would yield an increase in the multi-locus genotype error rate from 4.9% to 13.1% (Eq. 6). The consequence of such elevated error rates would be a significant increase in the total number of missed matches (i.e., samples from the same individual which fail to match; Paetkau 2003). Missing matches, in

TABLE 4. Extended.

Second-order relatives			Unrelated individuals		
Matches/pairs		Fraction of total matches	Matches/pairs		Fraction of total matches
No.	Fraction		No.	Fraction	
18/8344	0.002	0.234	55/79 800	0.001	0.714
2/7391	0.0003	0.667	1/71 786	0.00001	0.333
66/8303	0.008	0.293	152/79 421	0.002	0.676
6/7919	0.001	0.667	3/76 085	0.00004	0.333
1/7840	0.0001	0.333	2/75 347	0.00003	0.667
1/7760	0.0001	1.000	0/74 618	0	0
82/8410	0.010	0.229	258/80 572	0.003	0.721
8/8038	0.001	0.258	21/77 195	0.000	0.677
2/7911	0.0003	0.500	2/76 089	0.00003	0.500
264/8448	0.031	0.224	890/80 958	0.011	0.755
41/8448	0.005	0.281	94/80 958	0.001	0.644
8/8448	0.001	0.400	9/80 958	0.0001	0.450
1/8448	0.0001	0.200	2/80 958	0.00002	0.400
0/8448	0	0	1/80 958	0.00001	1.000

TABLE 5. Expected and observed number of matches treating all pairs of individuals as unrelated.

Locus	$I_{[UR]}$	Pairs	Matches expected	Matches observed	Observed : expected
GATA417	0.027	89 253	2410	2579	1.07
EV037	0.031	89 253	2810	2585	0.92
GATA053	0.048	81 003	3866	3854	1.00
RW004-10	0.052	88 410	4610	4364	0.95
GT011	0.053	89 676	4742	4698	0.99
GGAA520	0.055	86 320	4711	4670	0.99
EV096	0.055	88 410	4906	4753	0.97
TAA031	0.056	88 831	5009	4940	0.99
GT211	0.058	89 676	5199	4653	0.89
GT023	0.064	89 676	5703	5687	1.00
GT015	0.067	86 320	5801	5980	1.03
GT101	0.095	88 831	8438	8590	1.02
GT575	0.10	90 100	8975	8895	0.99
GATA098	0.14	89 676	12 410	12 330	0.99
GT307	0.14	90 100	12 740	12 556	0.99
EV094	0.15	90 100	13 730	13 371	0.97
GT195	0.19	90 100	17 150	17 634	1.03
GT271	0.20	90 100	18 370	18 878	1.03
EV001	0.22	90 100	19 510	19 345	0.99
GATA028	0.30	88 831	26 510	25 649	0.97
Average				9301	0.99
Discrepancy				−106	

Notes: See *Materials and methods* for a definition of *I*. Discrepancy is the difference between the expected and observed number of matches.

turn, will result in an upward bias of mark–recapture estimates of abundance, leading to the conclusion that a population is larger than is truly the case (Mills et al. 2000, Waits and Leberg 2000, Creel et al. 2003, Roon et al. 2005). Accordingly, increasing the number of loci unnecessarily may in effect increase endangerment (i.e., the population is actually smaller than a genetic tagging study leads one to believe). There are a number of ways to minimize genotyping error and missing matches, which have been dealt with in several extensive reviews (Bonin et al. 2004, Pompanon et al. 2005, Waits and Paetkau 2005).

Is it then really necessary to use the full-sibling (FS) criterion? The studies originally offering this suggestion (Woods et al. 1999, Waits et al. 2001) did not include an actual assessment of the effect of close relatives among their samples, but inferred that close relatives were the cause of the higher-than-expected matching rates.

The samples used as the basis for our assessment include samples that were collected from a summer feeding aggregation of maternally related humpback whales; thus they represent the kind of case that Waits et al. (2001), Woods et al. (1999), and later Paetkau (2004) suggested could be problematic. Our estimation of relatedness among the sampled individuals did reveal (as expected from the sighting history) a number of individuals inferred to be close relatives. However, the largest proportion of relatives stemmed from dyads inferred to be second-order relatives, which accounted for 9.4% of all dyads. In contrast, the percentage of dyads inferred as first-order relatives was estimated at only 0.3% of all dyads. Consequently, even though $I_{[SIBS]}$ exceeds $I_{[UR]}$ by approximately one order of magnitude in this study, the number of dyads of individuals that were inferred as first-order relatives is several orders of magnitude lower. Accordingly, the contribution of false matches from dyads of first-order relatives to the total number of matches becomes small, despite the substantially higher *I* values. Although our study did not reveal any major discrepancy between the average expected and observed number of random matches when basing the calculation upon $I_{[UR]}$, our results did show that dyads inferred as second-order relatives contributed substantially to the number of random (false) matches. Our four multi-locus datasets represented an opportunity to assess when multi-locus genotypes contained too few loci to distinguish among different individuals (Table 4). These data sets represent the cases Paetkau (2003) refers to as MM-1, MM-2, and so forth, denoting samples that mismatch at one, or two loci, respectively. At MM-1 and MM-2, only a few matches were observed between different individuals (one to four matches; Table 4) and these were evenly distributed between dyads inferred as composed of unrelated individuals or second-order relatives. No matches were recorded for inferred dyads of first-order relatives at the full complement of loci for each set (Table 4). Dyads inferred as second-order relatives were responsible for an average of 15% of the matches observed at single loci (Table 3) and between 100% and 0% for MM-1 loci (Table 4). Thus, in our study it appears that dyads inferred as first-order relatives were present at insufficient rates to add any erroneous matches at M-1. In contrast, matches were observed between individuals inferred as second-order relatives or unrelated individuals (Table 4).

TABLE 6. Fractions of different levels of relationships inferred from subset of the data.

	Parent and offspring		Full siblings		Second-order relatives		Unrelated	
Set	Obs.	Fraction	Obs.	Fraction	Obs.	Fraction	Obs.	Fraction
01	7579	0.084	4957	0.055	11 503	0.128	66061	0.733
02	3558	0.039	2126	0.024	14 459	0.160	69921	0.776
03	8562	0.095	3254	0.036	12 500	0.139	65784	0.730
04	6098	0.068	2972	0.033	13 356	0.148	67674	0.751
Average	6449	0.072	3336	0.037	12 955	0.144	67360	0.748
All 20 loci	154	0.002	117	0.001	8491	0.094	81338	0.903

Note: Obs. is the number of observations; fraction is the proportion of observations showing close relationships.

So which I to aim for? Our study indicated that the FS criterion is overly conservative, a point raised earlier by other authors from comparatively small data sets (Paetkau 2003, McKelvey and Schwartz 2004, Paetkau 2004). In fact, in our case, $I_{[UR]}$ appeared to predict the observed number of matches well, even in a sample that contained a large proportion of known and inferred dyads of related individuals. For most applications of genetic tagging, the required increase in effort to satisfy what may be an overly conservative FS criterion is substantial and is likely to result in an increase in the number of missed matches, potentially rendering some studies infeasible. In essence, the issue comes down to the balance between the relative proportions of the different relationship categories in the sample and the relative difference in probability of identity. For instance, if $I_{[2ND]}$ is 10 times higher than I, but the expected proportion of dyads of second-order relatives in a sample is 100 times lower than the proportion of dyads of unrelated individuals, then the contribution of second-order relatives to the total number of incorrectly matching samples would be at one in 10. Consequently, if the number of loci to be genotyped has been based upon I and the expected number of dyads of unrelated individuals has been estimated at less than one, then the likelihood of any falsely matching second-order relatives will be very low. The expected proportion of different relatedness categories in a random population sample may be estimated using demographic modeling (for an example, see Peery et al. 2008). Importantly, if only a small proportion of the population has been sampled (and the sample is representative), then the likelihood of sampling any dyads of close relatives will be small, and any concerns about falsely matching relatives can safely be ignored.

However, in some cases there may be concerns that the sampling is unrepresentative to an unknown degree, or vital demographic parameters are unknown, making it infeasible to make any predictions about the expected proportions of different relatedness categories in a sample of individuals. In such cases, the presence of close relatives might present an issue. There are several different ways to resolve this issue: (1) implement a two-step approach; (2) estimate the fraction of first- and second-order dyads, calculate the expected number of matches, and adjust the number of loci accordingly; or (3) use $I_{[2ND]}$ to guide the choice and number of loci.

A two step-approach reduces the required experimental effort as well as the overall error rate as much as possible, while still maintaining the high degree of statistical rigor offered by using $I_{[SIBS]}$ of the identification of individuals. The two steps are; (1) genotype samples using $I_{[UR]}$ (which may be estimated once a reasonable number of samples have been genotyped) as the initial guideline; and then (2) genotype additional loci in only those samples with matching genotypes to satisfy the FS criterion.

Such a dynamic two-step approach would greatly reduce the overall effort and the overall multi-locus error rate (due to many fewer genotypings), because the total number of pairwise comparisons is greatly reduced but the stringency of the genetic tagging would still be at the level of the FS criterion.

Another strategy is to take advantage of the (albeit reduced) information regarding relatedness that is contained even in a handful of microsatellite loci. Although estimates of relationships become increasingly imprecise with decreasing data, estimates based upon the preliminary genotyping results should provide a ballpark figure of the relative proportions of different relationships among the sampled individuals, which in turn may be used to guide the decision of how many additional loci will be needed to avoid erroneously matching close relatives. Table 6 lists the estimates of relationships inferred from each of the four subsets of microsatellite genotypes used in our estimations. In all four cases, the estimated fraction of close relatives among the analyzed samples supersedes that inferred on the full set of 20 microsatellite loci by as much as 0.15 (on average 0.75 vs. 0.90 for all 20 microsatellite loci). This bias toward a too-high fraction of close relatives is preferable over the opposite (i.e., a too-low fraction of close relatives), as the bias will guide a study toward genotyping an upward-biased number of loci.

Finally, our study suggests that second-order relatives contribute substantially to the total number of matches. Hence, it might be reasonable to use $I_{[2ND]}$ to guide the decision of how many loci to genotype if a study is similar to ours in terms of the biology of the target

PLATE 1. A researcher prepares to collect a skin biopsy sample from a humpback whale for molecular genetic analyses. Photo credit: Provincetown Center for Coastal Studies, NOAA permit #663-1778.

species and likely sampling composition. In our study, such a strategy would increase the number of loci from five (using $I_{[UR]}$) to eight, which compares favorably to the 14 loci required to satisfy the FS criterion.

Exactly which approach is preferable depends to a large extent upon specific context, such as the demographic characteristics of the target species, litter size and mating system, proportion of population sampled, and any other factors affecting how many dyads of close relatives are contained in the population sample. Finally, the specific conservation goals and resources may also determine what kind of bias is most preferable, such as (1) avoiding incorrect matches (i.e., between close relatives), which requires a substantial laboratory effort to genotype sufficient loci at low error rates, or (2) accepting some low level of incorrect matches, which would reduce the laboratory effort but result in downward-biased capture–mark–recapture estimates of abundance and upward estimates of movement rates.

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