

A comparison of several measures of genetic distance and population structure with microsatellite data: bias and sampling variance

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Abstract: Because of their rapid mutation rate and resulting large number of alleles, microsatellite DNA are well suited to examine the genetic or demographic structure of fish populations. However, the large number of alleles imply that large sample sizes are required for accurate reflection of genotypic frequencies. Estimates of genetic distance are often biased at small sample sizes, and biases and sampling variances can be affected by the number of, and distances between, alleles. Using data from a large collection of larval cod (*Gadus morhua*) from a single area, I examined the effect of sample size on seven genetic distance and two structure metrics. Pairs of samples (equal or unequal) of various sizes were drawn at random from a pool of 856 individuals scored for six microsatellite loci. $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} were the best performers in terms of bias and variance. Sample sizes of $50 \leq N \leq 100$ individuals were generally necessary for precise estimation of genetic distances and this value depended on number of loci, number of alleles, and range in allele size. $(\delta\mu)^2$ and D_{SW} were biased at small sample sizes.

Résumé : Parce qu'ils sont le siège de mutations rapides, ce qui se traduit par un nombre d'allèles élevé, les microsatellites de l'ADN conviennent bien à l'étude génétique et démographique des populations de poissons. Toutefois, étant donné le nombre élevé d'allèles en jeu, il faut des échantillons à effectif élevé pour que l'évaluation des fréquences génotypiques soit exacte. L'estimation de la distance génétique est souvent biaisée avec les échantillons de faible effectif; en outre, le nombre d'allèles et la distance qui les sépare peuvent influencer sur le biais et sur la variance d'échantillonnage. Nous avons utilisé des données portant sur un vaste ensemble de larves de morue (*Gadus morhua*) provenant d'une même zone pour étudier l'effet de l'effectif des échantillons sur sept distances génétiques et deux paramètres de structure. Nous avons choisi au hasard des paires d'échantillons (égaux ou inégaux) d'effectifs variés dans un ensemble constitué de 856 spécimens présentant six microsatellites. $(\delta\mu)^2$, D_{SW} , R_{ST} et F_{ST} ont donné les meilleurs résultats (biais et variance). Nous avons constaté qu'en général, il faut des échantillons d'au plus 50 sujets et d'au moins 100 sujets ($50 \leq N \leq 100$) pour estimer avec précision les distances génétiques, la valeur de l'effectif dépendant du nombre de locus, du nombre d'allèles et de la variation de la taille des allèles. $(\delta\mu)^2$ et D_{SW} présentaient un biais avec les échantillons de faible effectif.

[Traduit par la Rédaction]

Introduction

The term microsatellites refers to the repetitive arrays of short nucleotide motifs containing di-, tri-, or tetra-nucleotides (Wright 1993; Jarne and Lagoda 1996). Microsatellite loci are abundant and widely distributed throughout the eukaryotic genome and often exhibit high levels of allelic polymorphism. Allelic variants, which are inherited in a Mendelian fashion, are believed to be selectively neutral (see Jarne and Lagoda 1996). Each microsatellite locus is flanked by a unique sequence to which complementary primers can be synthesised. The microsatellite loci can then be isolated with relative ease by PCR amplification from minute quantities of fresh or preserved tissue. These qualities of microsatellites make them very useful as genetic markers for studies of population differentiation and stock identification (reviewed in Park and Moran 1994; Wright and Bentzen 1994; O'Reilly and Wright 1995), of kinship and parentage exclusion (Amos et al. 1993; Queller et al. 1993; Morin et al. 1994; Kellogg et al. 1995), and of genome mapping (Hearne et al. 1992).

Microsatellites have already been used in population studies of humans (e.g., Deka et al. 1991, 1994; Edwards et al. 1992; Bowcock et al. 1994; Clark et al. 1995), mammals (e.g., Roy et al. 1994; Paetkau et al. 1995), and fish (e.g., Bentzen et al. 1996; Ruzzante et al. 1996a, 1996b, 1997; McConnell et al. 1997).

The large number of allelic variants present in most microsatellite loci (e.g., Edwards et al. 1992; Brooker et al. 1994; Ruzzante et al. 1996a), however, requires that large sample sizes (i.e., substantially in excess of 50 individuals) are needed to accurately reflect genotype frequencies, especially if many alleles are present at low frequencies (Carvalho and Hauser 1994). A related problem is that some of the measures of genetic distance used in population genetic studies are biased (i.e., they show a systematic departure of the estimated mean from the true or expected value) at small sample size. Thus, the precise estimation of genetic distances and population structure requires knowledge of minimum sample sizes above which the influence of bias and sampling variance is minimized.

I explored the relationship between sample size and a number of genetic distance and population structure metrics using information from up to six microsatellite DNA loci scored in over 1300 Atlantic cod (*Gadus morhua*) larvae collected from a single area in the Northwest Atlantic (Ruzzante

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et al. 1996a). I examined the influence of sample size on bias and sampling variance for nine estimators: average squared distance (ASD) and $(\delta\mu)^2$ (Goldstein et al. 1995a, 1995b); D_{SW} (Shriver et al. 1995); allele sharing (Bowcock et al. 1994); Rogers' (1972) genetic distance, Cavalli-Sforza and Edwards' (1967) chord distance, and Nei et al.'s (1983) genetic distance; R_{ST} (Slatkin 1995); and F_{ST} (Wright 1951). The performance of several of these genetic measures has recently been examined with simulated data by a number of researchers, notably Goldstein et al. (1995a, 1995b), Shriver et al. (1995), Slatkin (1995), and Takezaki and Nei (1996). These studies focused primarily on the question of which genetic measure most accurately reflects (i) the correct topology of a simulated phylogenetic tree and (ii) the evolutionary time since population divergence. However, because of the combination of high mutation rates compared with allozymes (Weber and Wong 1993; Jarne and Lagoda 1996) and constraints in allele size (Garza et al. 1995), microsatellites are thought to be most useful for studies of demographic structure or those involving a very short time perspective (Nauta and Weissing 1996). In the present paper, I focused on the question of what is the minimum sample size required to detect demographic structure, such as boundaries among spawning components in a stock or population complex. This is a primary concern among fisheries researchers who are often asked to provide management advice concerning fish populations that may or may not mix regularly during spawning and that may or may not interbreed. To examine the minimum sample size required to detect demographic structure I used microsatellite DNA data from fish (Atlantic cod), which have substantially more alleles per microsatellite locus than most other major groups studied thus far (Brooker et al. 1994; see also Bentzen et al. 1996; Ruzzante et al. 1996a; McConnell et al. 1997). I examined how the performance (i.e., bias and variance) of the nine metrics was affected by sample size and differences in sample size, number of loci, number of alleles at a locus, and maximum divergence among alleles.

Materials and methods

Sample collections and laboratory procedures

Larval cod samples were collected from one location on the Scotian Shelf in the Northwest Atlantic. The larvae (1337 individuals) were collected from a large larval aggregation detected within a gyrelike water mass tracked for 3 weeks in November and December 1992 on the Scotian Shelf (Griffin and Lochmann 1993). The resident cod larvae within the tracked water mass were sampled continuously every 4–8 h during this period. Further details of the field procedures are available elsewhere (Taggart et al. 1996; Lochmann et al. 1997; Ruzzante et al. 1996a).

Tissue collections and processing

Cod larvae initially preserved in liquid nitrogen were thawed, and the eyes were removed for genetic analysis. The eyes were placed in 50 μ L of extraction buffer (10 mM Tris (pH 8.3) containing 50 mM KCl and 0.8% Tween 20) and were held at -20°C . The DNA was released from the tissue by digestion with proteinase K (200 μ g/mL final concentration) at 65°C overnight. Following digestion, the samples were incubated at 95°C for 15 min to deactivate the proteinase K, and 1–2 μ L of this mixture was used directly for PCR. Further details on processing are available elsewhere (Ruzzante et al. 1996a).

Genetic analysis

PCR analysis, including reaction conditions, was conducted as previously described by Brooker et al. (1994). Six sets of cod microsatellite primers were used: Gmo2, Gmo132, and Gmo145 (Brooker et al. 1994); Gmo4 (Wright 1993); and Gmo141 and Gmo120 (Ruzzante et al. 1996a, 1996b, respectively). Probes Gmo2, Gmo4, Gmo120, Gmo132, and Gmo141 are perfect GT repeats, and Gmo145 is a compound $G_x(GA)_x$ repeat as defined by Tautz (1989). For all loci except Gmo145, the allele sizes varied by increments of two base pairs (bp). Gmo145 showed occasional alleles that differed by a single base-pair change. The PCR products were resolved on 6.5% sequencing gels, and the alleles were sized relative to a sequence ladder generated from M13mp18 (Yanisch-Perron et al. 1985).

Genetic distance measures and data analysis

I examined how a number of factors such as number of loci, number of alleles at each locus, and distance between alleles (measured in base pairs) interacted with sample size to affect both bias and sampling variance of a variety of traditional and recently developed, microsatellite-specific measures of genetic distance and population structure. Most of these measures were used in our recent studies on Atlantic cod (Bentzen et al. 1996; Ruzzante et al. 1996a, 1996b, 1997) and Atlantic salmon (*Salmo salar*; McConnell et al. 1997) genetic structure. Here I present the definition and a brief explanation for each of the measures I examined.

Rogers' (1972) genetic distance (D_R) is

$$D_R = \frac{1}{2l} \sum_j \left(\sum_i^{m_j} (x_{ij} - y_{ij})^2 \right)^{1/2}$$

where l is the number of loci examined, x_{ij} and y_{ij} are the frequencies of the i th allele on the j th locus in populations x and y , respectively. Summation is thus over all alleles within a locus and then over all loci.

Cavalli-Sforza and Edwards' (1967) chord distance (D_C) is

$$D_C = \frac{2}{\pi l} \sum_j \left(2 \left(1 - \sum_i^{m_j} (x_{ij} - y_{ij})^{1/2} \right) \right)^{1/2}$$

where l , x_{ij} , and y_{ij} are as for D_R above.

Nei et al.'s (1983) D_A distance is

$$D_A = 1 - \frac{1}{l} \sum_j \sum_i^{m_j} (x_{ij} y_{ij})^{1/2}$$

where again, l , x_{ij} , and y_{ij} are as for D_R above. The previous three measures are essentially geometric representations on multidimensional hyperspaces of differences in allele frequencies between populations and are thus independent of any mutational model (Takezaki and Nei 1996).

Allele sharing distance (Bowcock et al. 1994) in this study was defined as $1 - P$, where P is the proportion over all loci of alleles shared between populations.

The remaining three genetic distances were all derived specifically for microsatellite loci and are based on the assumption that alleles mutate following the single-step mutational model (Shriver et al. 1993; Valdes et al. 1993; Di Rienzo et al. 1994, and references therein; Goldstein et al. 1995a, 1995b; Slatkin 1995). Average squared distance (Goldstein et al. 1995a) is

$$\text{ASD} = \sum_i \sum_j (i - j)^2 x_i y_j$$

where summation is over the squared difference in repeat number of all alleles i in population x and all alleles j in population y , and x_i and y_j are the frequencies of the i th and j th allele in populations x and y , respectively.

Goldstein et al.'s (1995b) $(\delta\mu)^2$ is

$$(\delta\mu)^2 = \sum_j^r (\mu_x - \mu_y)^2$$

where μ_x and μ_y are the average allelic states (mean allele size in repeat number) in populations x and y , respectively, and summation is over all loci r . The expectation for $(\delta\mu)^2$ is

$$E(\delta\mu)^2 = E(\bar{x} - \bar{y})^2 = \left(\sum \frac{i}{n} - \sum \frac{j}{m} \right)^2$$

where i and j are, as for ASD above, the length in repeat number of all alleles i in population x and all alleles j in population y , respectively, and n and m are sample sizes for populations x and y , respectively. It is relatively straightforward to show that

$$E(\delta\mu)^2 = \frac{\sigma_x^2}{n} + (\mu_x - \mu_y)^2 + \frac{\sigma_y^2}{m}$$

which indicates that $(\delta\mu)^2$ is biased and that the bias is dependent on the variance in allele size and decreases with increasing sample size.

Shriver et al.'s (1995) stepwise weighted genetic distance is an extension of Nei's (1972) minimum genetic distance that weighs the product of allele frequencies (within and between populations) by the absolute value of the difference in mutational steps between any two alleles (assuming single-step mutations) measured in base pairs (Shriver et al. 1995):

$$D_{SW} = d_{xyw} - \frac{d_{xw} + d_{yw}}{2}$$

where

$$\hat{d}_{xw} = 2n_x \sum \sum \hat{x}_i \hat{x}_j \delta_{ij} / (2n_x - 1)$$

$$\hat{d}_{yw} = 2n_y \sum \sum \hat{y}_i \hat{y}_j \delta_{ij} / (2n_y - 1)$$

$$\hat{d}_{xyw} = \sum_i \sum_j \hat{x}_i \hat{y}_j \delta_{ij}$$

$$\delta_{ij} = |i - j|$$

and where \hat{x}_i and \hat{x}_j are observed frequencies of alleles i and j in population x , \hat{y}_i and \hat{y}_j are observed frequencies of alleles i and j in population y , n_x and n_y are the number of individuals sampled in populations x and y , respectively, and δ_{ij} is the absolute value of the difference in mutational steps between alleles i and j measured in base pairs.

Finally, I also examined the performance of two measures of population structure, Slatkin's (1995) R_{ST} and the traditional F_{ST} measure (Wright 1951). Slatkin's (1995) R_{ST} was calculated as

$$R_{ST} = \frac{\bar{S} - S_w}{\bar{S}}$$

where \bar{S} is the average of the estimated variances of allele sizes in the collection of populations together, and S_w is the average of the estimated variances within each population (Slatkin 1995); thus, R_{ST} is the fraction of the total variance that is between populations. This ratio of variances approach is exactly equivalent to Slatkin's analytical derivation (Slatkin 1995, p. 458, eqs. 9a, 9b, and 10) only when sample sizes are all equal. In most studies of natural populations, however, sample sizes will differ between populations and in such cases populations with larger sample sizes will contribute more to the total variance than populations with smaller sample sizes (Goodman 1997). When sample sizes differ, R_{ST} can be calculated following a conventional analysis of variance (ANOVA) framework as suggested by Michalakis and Excoffier (1996) and Goodman (1997). Here, I followed Goodman's formulation (Goodman 1997, table 1) as well as the ratio of variances approach to calculate R_{ST} when sample sizes differ. Finally, F_{ST} was estimated following Weir and Cockerham (1984). Estimates of R_{ST} and F_{ST} combined over loci were calculated

by first averaging numerators and denominators across loci and then taking ratios, as suggested by Slatkin (1995) and Weir and Cockerham (1984), respectively.

Effect of balanced and unbalanced sampling

Estimates were obtained between pairs of samples that were equal in size and between pairs of samples that differed in size. Samples of various sizes were drawn at random with replacement from a pool of 856 larvae for which there was information on all six microsatellite loci. Individuals were assigned to one of two groups. Thus, the bootstrap procedure consisted of shuffling entire six-loci genotypes among sample populations. This is a more conservative approach than shuffling individual loci independently because, for some measures, it produces slightly larger confidence intervals (data not shown). Sample sizes when both sizes were equal were 10, 20, 40, 50, 60, 80, 100, 150, 200, and 250 individuals. When sample sizes differed, samples varied in size by a factor of between four and five and effective average sample sizes ($N_e = N - (\sum n_i^2 / N)$) were comparable with those when sample sizes were equal. I compared samples of 6 and 30 individuals ($N_e = 10$), 12 and 60 ($N_e = 20$), 25 and 100 ($N_e = 40$), 31 and 130 ($N_e = 50$), 37 and 159 ($N_e = 60$), 50 and 200 ($N_e = 80$), 60 and 300 ($N_e = 100$), 93 and 400 ($N_e = 150$), 126 and 490 ($N_e = 200$), and 158 and 600 ($N_e = 250$).

The procedure was repeated 1000 times (trials) for each sample size or combination. Genetic distances and estimates of population structure were evaluated for each trial and then averaged over the 1000 trials. Confidence intervals were evaluated by taking the difference between the 2.5th and the 97.5th percentiles of the empirical distribution for each metric.

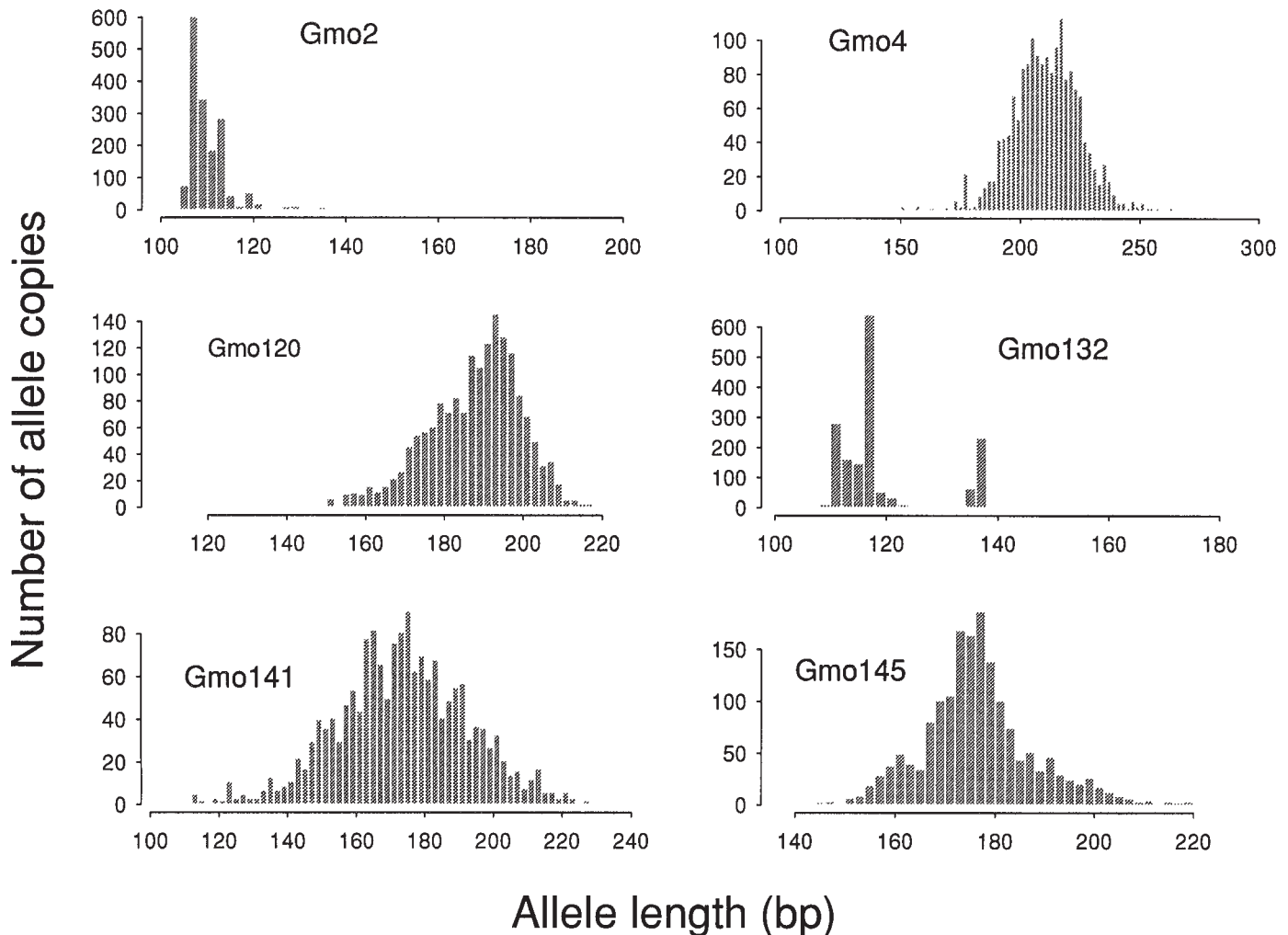
Effect of number of loci

To examine the effect of the number of loci, I compared results obtained with two and six loci. The two loci used in this set of comparisons were Gmo2 and Gmo4. These loci were chosen because they represented the broad range in number of alleles available; i.e., Gmo2 had the lowest (i.e., 29) and Gmo4 had one of the largest (i.e., 61) number of alleles (Table 1).

Effect of allele size and number

For all estimates I examined the effect of sample size on bias and sampling variance as a function of number of alleles and distance (in number of base pairs) between alleles. To do this I binned alleles at different loci following a "floating" bin approach. Alleles at Gmo2 and Gmo132 were binned in two different ways. First, neighbouring alleles were binned to reduce their number to 10 alleles at each locus with a distance between the longest and shortest allele of 82 bp (Gmo2, 10 alleles, 82 bp; Gmo132, 10 alleles, 82 bp). Second, alleles at these same two loci (i.e., Gmo2 and Gmo132) were binned to obtain 25 alleles with a distance between the longest and shortest allele of 68 bp (Gmo2, 25 alleles, 68 bp; Gmo132, 25 alleles, 68 bp). Alleles at Gmo4 and Gmo141 were also binned in two different ways. First, alleles were binned to reduce their number to 30 at each locus with a distance between longest and shortest allele of 90 bp (Gmo4, 30 alleles, 90 bp; Gmo141, 30 alleles, 90 bp). Second, alleles were binned to reduce their number to 25 with a distance between longest and shortest allele of 138 bp (Gmo4, 25 alleles, 138 bp; Gmo141, 25 alleles, 138 bp). Comparison of estimates obtained with the 2 loci with 10 alleles separated by a maximum of 82 bp with estimates obtained with the 2 loci with 30 alleles separated by a maximum of 90 bp allowed examination of the effect of number of alleles while controlling for maximum distance between alleles, because this distance was roughly equivalent in both data sets. Similarly, comparison of estimates obtained with the 2 loci with 25 alleles separated by a maximum of 68 bp with estimates obtained with the 2 loci with 25 alleles separated by a maximum of 138 bp allowed examination of the effect of distance between alleles while controlling for number of alleles.

Fig. 1. Allele counts histograms for six Atlantic cod (*Gadus morhua*) microsatellite DNA loci: Gmo2, Gmo4, Gmo120, Gmo132, Gmo141, and Gmo145.



Results

Single-locus analysis and allele frequency distribution

All six microsatellite loci examined were highly polymorphic (Fig. 1). The number of alleles detected per locus in the subset for which there was information on all six loci ($N = 856$) varied between 26 for Gmo2 and 61 for Gmo141 (44 ± 14 (mean \pm SD); Table 1). For most loci, however, a large percentage of these alleles were observed at very low frequencies, i.e., in general only up to about one half of the alleles were at frequencies higher than 0.01 (Table 1, Fig. 1). The total number of alleles observed and the number of alleles occurring at frequency greater than 1% increased only slightly when all individuals scored for each locus were examined (Table 1).

In the following sections I first explored the effect of number of loci on sampling variance (Fig. 2); then, using six loci I examined how differences in sample sizes affect sampling variance in pairwise comparisons (Figs. 2c', 2f', 2g', 2h', and 2i'). Subsequently, using sets of two loci (binned in different ways, see Fig. 3), I examined how number of alleles at a locus and distance between alleles affect small sample size

related bias in the various distance measures (Fig. 4). Finally, I explored the effect of each of these two factors (i.e., number of alleles and distance between alleles) on sampling variance (Figs. 5 and 6).

Number of loci

I explored the effect of the number of loci on all nine measures of genetic distance and population structure by comparing their sampling variances and expected values using two and six loci. Sampling variances for D_R , D_C , allele sharing, and ASD were little affected by number of loci and were always relatively large regardless of sample size (cf. Figs. 2a vs. 2a', 2b vs. 2b', 2d vs. 2d', and 2e vs. 2e'). Sampling variance for D_A (Fig. 2c vs. 2c') decreased moderately with increasing sample size but was relatively unaffected by number of loci. Four of these five measures, i.e., D_R , D_C , D_A , and allele sharing, exhibited strong small size related bias in their expectations (Figs. 2a–2d). ASD showed no bias (Fig. 2e), which is consistent with Goldstein et al. (1995a).

Sampling variances for $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} all decreased considerably with increasing number of loci and with

Table 1. Total number of alleles observed per locus in the data subset for which information was available on all six loci ($N = 856$) and in all individuals scored for each locus.

Probe*	All six loci		All individuals	
	No. of alleles	N	No. of alleles	N
Gmo2	26 [8]	856	29 [8]	1308
Gmo4	60 [26]	856	61 [26]	1001
Gmo120	43 [22]	856	44 [23]	1228
Gmo132	29 [8]	856	32 [9]	1316
Gmo141	61 [30]	856	63 [31]	1286
Gmo145	45 [24]	856	48 [24]	1232

Note: Numbers in square brackets are the numbers of alleles at individual frequencies >0.01 .

*Primers and PCR conditions are in Wright (1993), Brooker et al. (1994), and Ruzzante et al. (1996b).

increasing sample size. For these measures, sampling variances were relatively low at sample sizes of about 100 individuals (Figs. 2*f* vs. 2*f'*, 2*g* vs. 2*g'*, 2*h* vs. 2*h'*, and 2*i* vs. 2*i'*). The effect of number of loci on sampling variance is most apparent at relatively small sample sizes. These four genetic measures ($(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST}) exhibited remarkably less bias than most previous measures (see also below).

Different sample sizes

Results in the previous section reflect comparisons between pairs of samples of equal size. I next examined the effect of differing sample sizes in pairwise comparisons using six loci. Because of the relatively poor performance (i.e., relatively high variances despite large sample sizes) of D_R , D_C , allele sharing, and ASD when sample sizes were equal, I excluded these measures from further analysis to save space.

When sample sizes differed, sampling variances for D_A , $(\delta\mu)^2$, D_{SW} , and F_{ST} (Figs. 2*c'*, 2*f'*, 2*g'*, and 2*i'*, dotted lines) were similar in magnitude to the variances obtained with equal sample sizes, and thus, these measures appear to be unaffected by differences in sample size as long as the effective average sample size remains unchanged. For R_{ST} , however, sampling variances when sample sizes differed depended on how R_{ST} was calculated. With the ratio of variances approach (Fig. 2*h'*, broken lines), variances were much larger when sample sizes differed than when they were equal (Fig. 2*h'*, compare broken lines with error bars). Variances were much less affected by differences in sample size when R_{ST} was calculated following the ANOVA approach (Fig. 2*h'*, dotted lines), although they were still slightly larger than when sample sizes were equal, particularly at relatively small sample sizes (Fig. 2*h'*, compare dotted lines with error bars).

To summarize thus far, of the nine measures examined, only $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} experienced a decrease in their sampling variances with increasing number of loci and with increasing sample size. The sampling variance for another measure, D_A , decreased with sample size, but this decrease was only moderate and appeared to be unaffected by increasing number of loci. Differences in sample size did not affect sampling variances of D_A , $(\delta\mu)^2$, D_{SW} , and F_{ST} . However, differences in sample size did affect the sampling variance of R_{ST} when R_{ST} was calculated as a ratio of variances, but when R_{ST} was calculated within an ANOVA framework, this effect was negligible, particularly at relatively large sample sizes

($N \geq 50$). Most of the measures that exhibited large sampling variances were also biased at relatively small sample sizes.

In the next sections I examined the effect of number of alleles and of distance between alleles on the four measures that performed best thus far: $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} . Three of these distance estimates ($(\delta\mu)^2$, D_{SW} , and R_{ST} , but not F_{ST}) include a measure of differences in allele lengths in their formulation.

Number of alleles at a locus and distance between alleles

To examine the effect of number of alleles on bias and sampling variance I compared a set of two loci each with 10 alleles (Gmo2, 10 alleles, 82 bp; and Gmo132, 10 alleles, 82 bp; Figs. 3*a* and 3*b*) with a second set of two loci each with 30 alleles (Gmo4, 30 alleles, 90 bp; and Gmo141, 30 alleles, 90 bp; Figs. 3*c* and 3*d*). Maximum distances between alleles were similar in both data sets (82 and 90 bp, respectively), thus allowing examination of the effect of number of alleles independently of range in allele size. Conversely, to examine the effect of range in allele size, I compared two data sets that differed in the number of base pairs between the longest and shortest alleles but not in the number of alleles (Figs. 3*e* and 3*f* vs. 3*g* and 3*h*, respectively).

Expectation and bias

Both $(\delta\mu)^2$ and D_{SW} showed bias in their expectations at relatively small sample sizes (Figs. 4*a* and 4*b*), and in both cases the bias was directly related to number of alleles and to range in allele size. For both, $(\delta\mu)^2$ and D_{SW} , bias decreased rapidly with increasing sample size and reached values close to the asymptote at samples sizes ranging between 50 and 100 individuals. R_{ST} and F_{ST} exhibited no bias (Figs. 4*c* and 4*d*, compare scale of Y axis with Figs. 5 or 6), the small differences in expected values observed at very small sample sizes probably arising as a consequence of the particular allele frequency spectrum obtained in the different types of binning.

Sampling variance

Number of alleles

The effect of number of alleles on sampling variance varied with the genetic measure considered. For $(\delta\mu)^2$, D_{SW} , and R_{ST} , sampling variances increased with number of alleles (Figs. 5*a* vs. 5*a'*, 5*b* vs. 5*b'*, and 5*c* vs. 5*c'*). For F_{ST} , sampling variances decreased instead with increasing number of alleles (Figs. 5*d* vs. 5*d'*). In all cases, the effect of number of alleles on sampling variance decreased with increasing sample size (Fig. 5).

Distance between alleles

The effect of range in allele size on sampling variance also varied with the genetic measure considered. Sampling variance for $(\delta\mu)^2$ and D_{SW} increased with increasing range in allele size (Figs. 6*a* vs. 6*a'* and 6*b* vs. 6*b'*) whereas range in allele size had almost no effect on the sampling variance of F_{ST} (Figs. 6*c* vs. 6*c'*). F_{ST} does not include allele size in its formulation so this factor is expected to have no influence on F_{ST} 's sampling variance, and this is borne out by the data (Figs. 6*d* vs. 6*d'*) except perhaps at very small sample sizes, where the slightly larger sampling variances observed with a shorter distance between alleles (Fig. 6*d*) probably arose,

Fig. 2. Genetic distances and estimates of population structure as a function of sample size (N) when sample sizes are equal (all panels) and when sample sizes differ (dotted lines in c' , f' , g' , h' , and i' , and broken lines in h' (see below)). Left panels represent values based on polymorphism at two loci (i.e., Gmo2 and Gmo4; they were chosen for this analysis because they had the lowest (Gmo2) and one of the largest (Gmo4) numbers of alleles). Right panels represent values based on polymorphism at six loci. Genetic distances are (a) and (a') D_R (Rogers 1972); (b) and (b') D_C (Cavalli-Sforza and Edwards 1967); (c) and (c') D_A (Nei et al. 1983); (d) and (d') allele sharing; (e) and (e') average squared distance (ASD) (Goldstein et al. 1995a); (f) and (f') $(\delta\mu)^2$ (Goldstein et al. 1995b); (g) and (g') D_{SW} (Shriver et al. 1995); (h) and (h') R_{ST} (Slatkin 1995); and (i) and (i') F_{ST} (Wright 1951). The 95% confidence intervals were obtained as the 2.5th and 97.5th percentile of the empirical distribution of 1000 trials; for equal sample sizes, confidence intervals are indicated by error bars, and for different sample sizes, confidence intervals are indicated by dotted and (or) broken lines in c' , f' , g' , h' , and i' . Sample sizes differed by a factor of between four and five, but the effective average sample sizes ($N_e = N - (\sum n_i^2/N)$) were equivalent to those when sample sizes were equal. When sample sizes differed, R_{ST} was calculated as a ratio of variances (h' , broken lines) and following Goodman's (1997) ANOVA approach (h' , dotted lines).

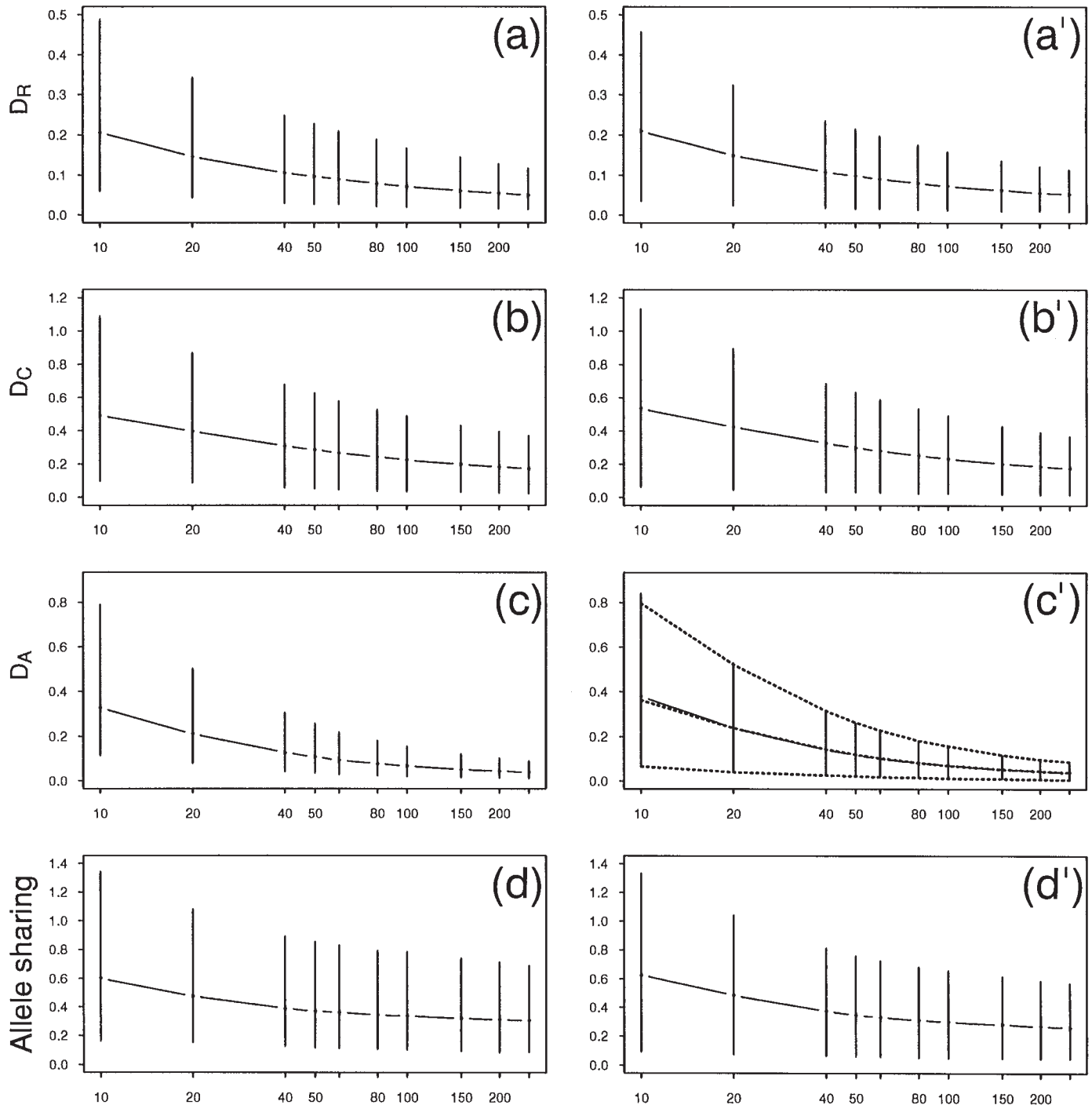


Fig. 2 (concluded).

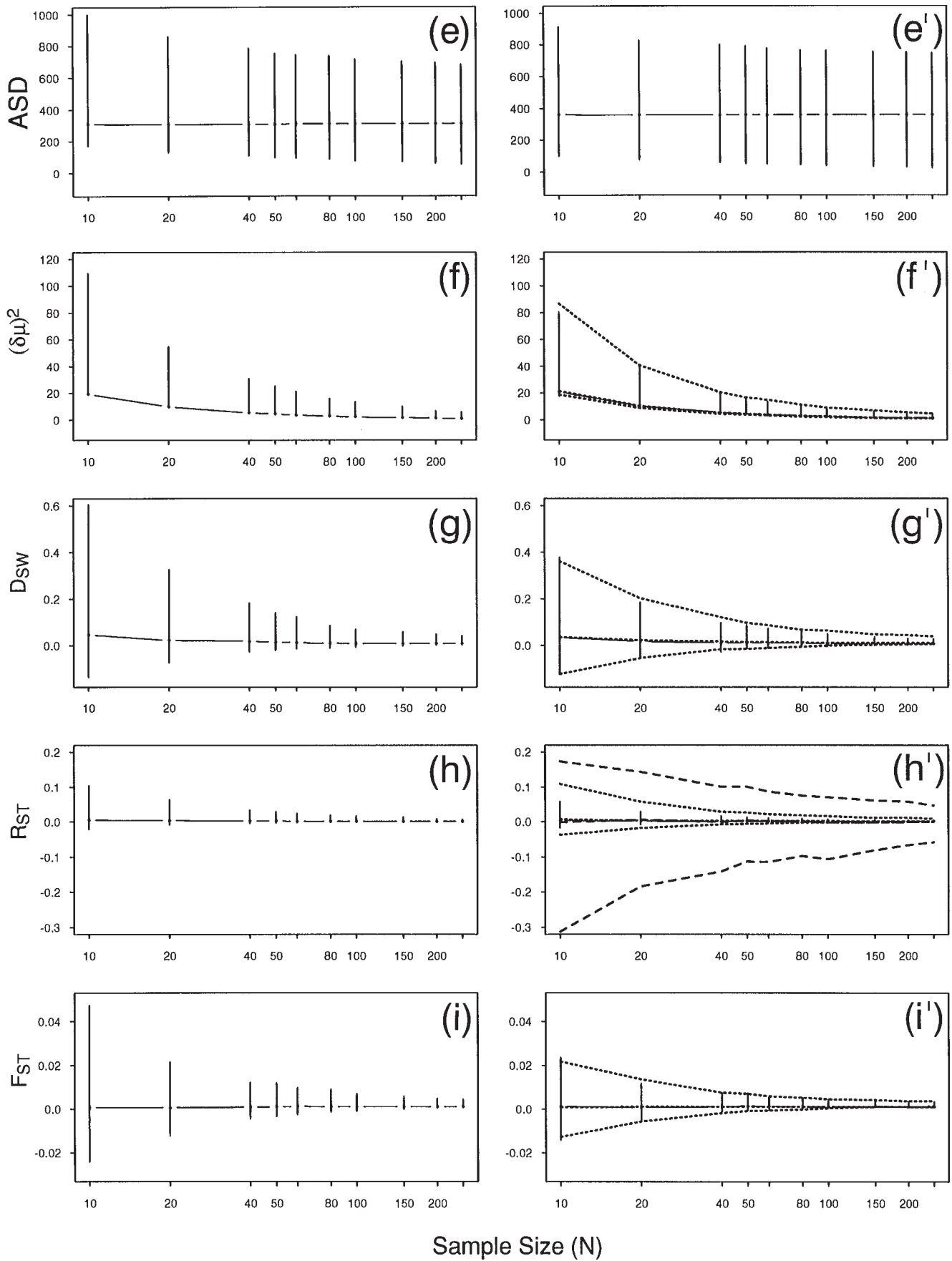


Fig. 3. Allele counts histograms for binned alleles. (a) Gmo2, 10 alleles, allele size range of 82 bp; (b) Gmo132, 10 alleles, allele size range of 82 bp; (c) Gmo4, 30 alleles, allele size range of 90 bp; (d) Gmo141, 30 alleles, allele size range of 90 bp; (e) Gmo2, 25 alleles, allele size range of 68 bp; (f) Gmo132, 25 alleles, allele size range of 68 bp; (g) Gmo4, 25 alleles, allele size range of 138 bp; (h) Gmo141, 25 alleles, allele size range of 138 bp.

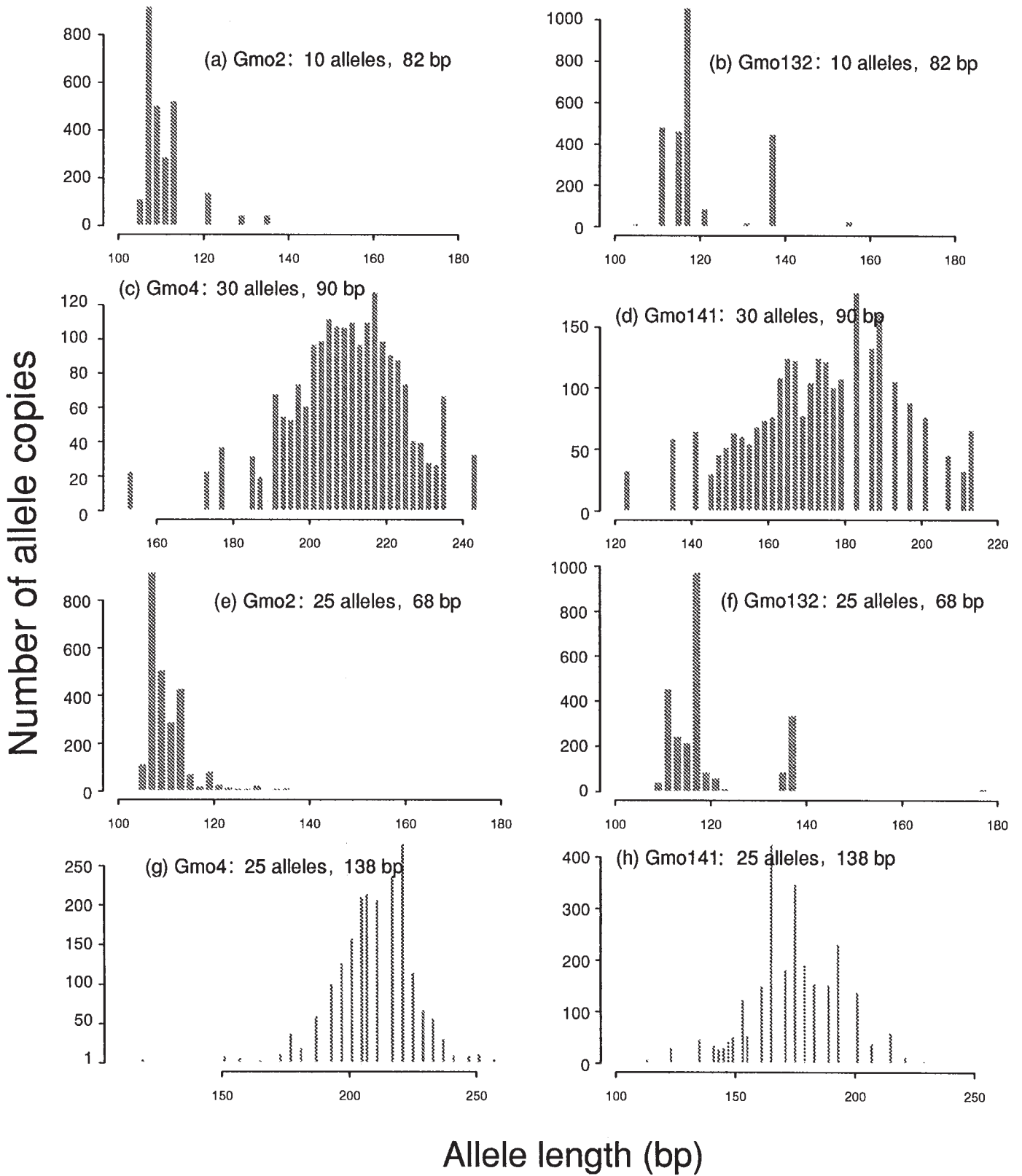
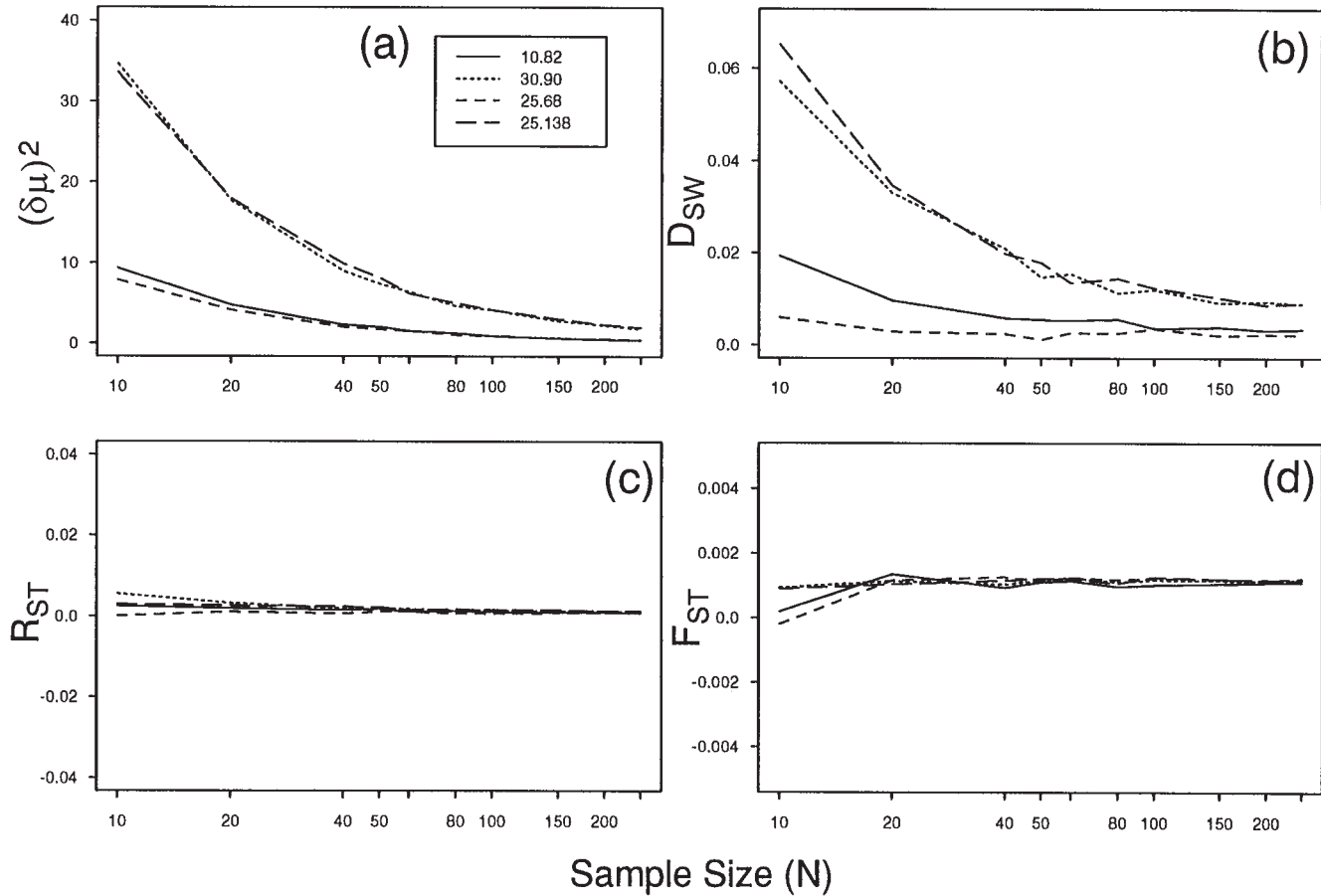


Fig. 4. Expected values of genetic distances and estimates of population structure as a function of sample size (N) when sample sizes are equal. All estimates are based on polymorphism at two loci as indicated in caption of Fig. 3. For the four panels, solid lines are Gmo2 and Gmo132, both with 10 alleles and a range in allele size of 82 bp; dotted lines are Gmo4 and Gmo141, both with 30 alleles and a range in allele size of 90 bp; broken lines with short dashes are Gmo2 and Gmo132, both with 25 alleles and a range in allele size of 68 bp; and broken lines with long dashes are Gmo4 and Gmo141, both with 25 alleles and a range in allele size of 138 bp. (a) $(\delta\mu)^2$, (b) D_{SW} , (c) R_{ST} , and (d) F_{ST} .



again, from the particular allele frequency distribution obtained with this particular set of binned loci.

Discussion

In this study I examined the influence of sample size on the performance of nine traditional and recently developed, microsatellite-specific measures of genetic distance and population structure. For each measure, I examined how number of loci, number of alleles at each locus, and range in allele size (measured in basepairs) interacted with sample size to affect bias and sampling variance. Overall, $(\delta\mu)^2$ (Goldstein et al. 1995b), D_{SW} (Shriver et al. 1995), R_{ST} (Slatkin 1995), and F_{ST} (Wright 1951) were the most useful measures (Figs. 2, 5, and 6) because they showed relatively low variances at moderate sample sizes, although there were limitations associated with each of them: (i) R_{ST} when estimated as a ratio of variances was sensitive to differences in sample size, but this effect became negligible when it was estimated following a conventional analysis of variance (ANOVA) approach as advocated by Michalakis and Excoffier (1996) and Goodman (1997) (Fig. 2); and (ii) $(\delta\mu)^2$ and D_{SW} showed bias at small sample sizes, and for both measures the bias was directly related to the

number of alleles and the range in allele size (Fig. 4). The magnitude of the bias relative to the sampling variance was larger for $(\delta\mu)^2$ than for D_{SW} (Figs. 5 and 6). The implications of these results are (i) when R_{ST} is used, sample sizes should be about equal, or else they should all be moderate to large ($N \geq 50$); and (ii) when $(\delta\mu)^2$ or D_{SW} are used and the number of alleles is relatively large ($N \geq 30$ alleles), sample sizes should also be relatively large ($N \cong 100$ individuals). Smaller sample sizes may be sufficient when there are fewer alleles per locus. However, regardless of which of $(\delta\mu)^2$, D_{SW} , R_{ST} , or F_{ST} is used, the cod microsatellite data analysed here suggest that sample sizes of N larger than 50, and preferably around 100, individuals are generally necessary for accurate (i.e., unbiased) and precise (i.e., small confidence intervals) estimation of genetic distances and population structure using microsatellite DNA markers. I next discuss the performance of these and the remaining measures in detail.

Most measures I examined, with the exception of ASD (Goldstein et al. 1995a), R_{ST} , and F_{ST} (estimated as θ following Weir and Cockerham 1984), exhibited extensive bias at small sample sizes of less than $N \cong 50$ individuals (Figs. 2 and 4–6), and all measures had relatively large confidence intervals associated with these small sample sizes (Figs. 2, 5,

Fig. 5. Effect of number of alleles. Genetic distances and estimates of population structure as a function of sample size (N) when sample sizes are equal. All values are based on polymorphism at two loci, as follows. Left panels: Gmo2 and Gmo132, both with 10 alleles each and a range in allele size of 82 bp. Right panels: Gmo4 and Gmo141, both with 30 alleles each and a range in allele size of 90 bp. (a) and (a') $(\delta\mu)^2$, (b) and (b') D_{SW} , (c) and (c') R_{ST} , and (d) and (d') F_{ST} . Error bars are 95% confidence intervals obtained as the 2.5th and 97.5th percentile of the empirical distribution of 1000 trials.

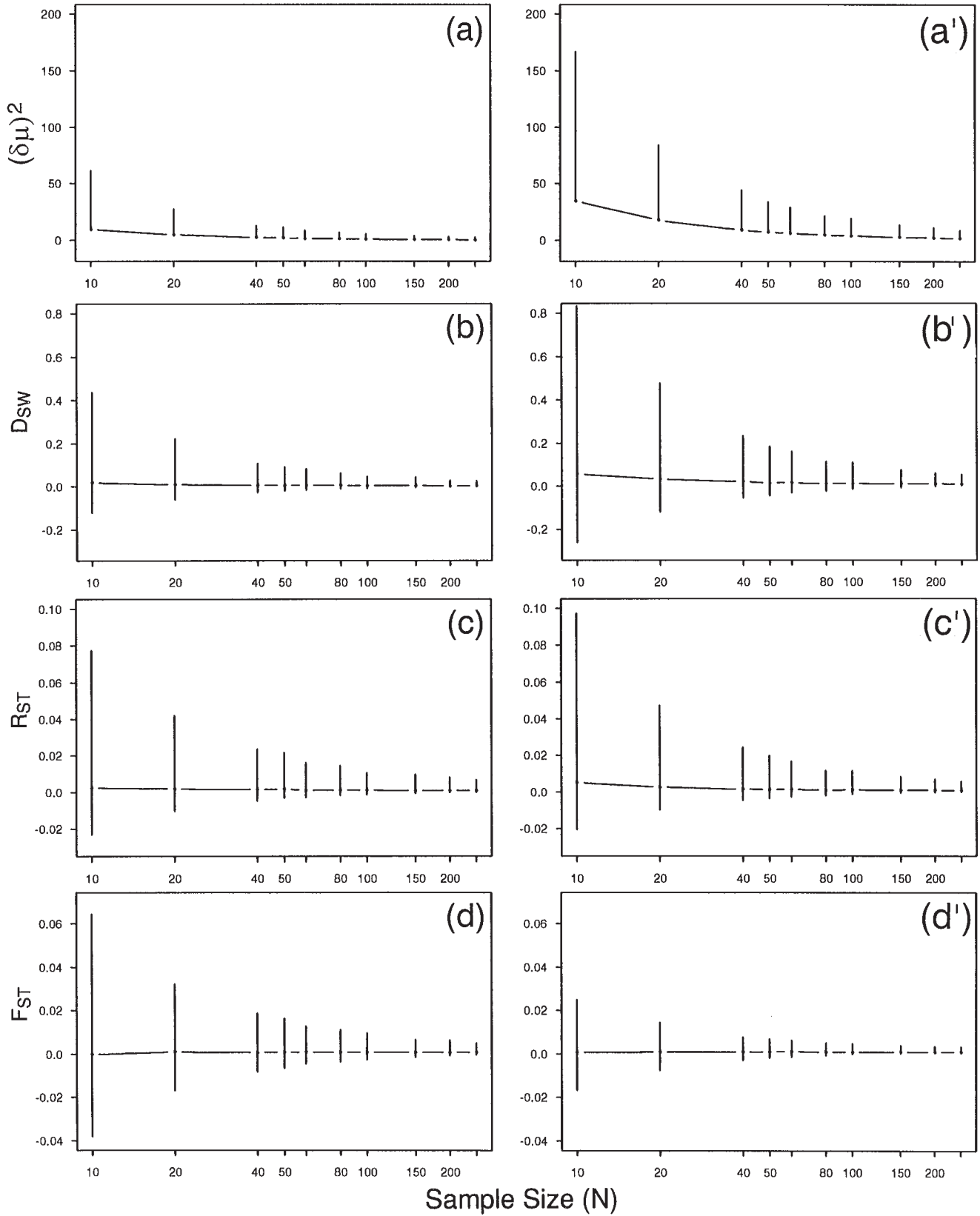
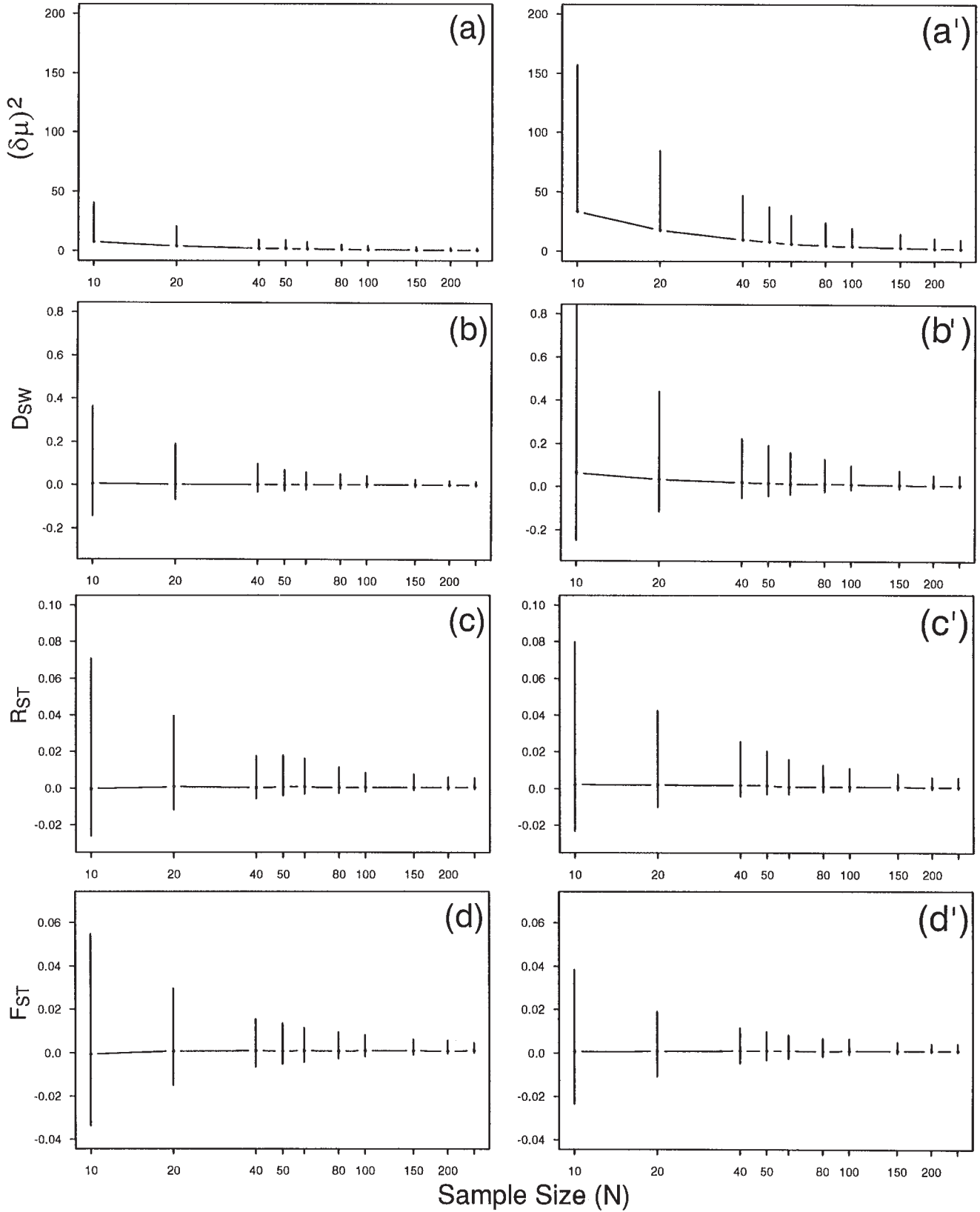


Fig. 6. Effect of distance between alleles. Genetic distances and estimates of population structure as a function of sample size (N) when sample sizes are equal. All values are based on polymorphism at two loci. Left panels: Gmo2 and Gmo132, both loci with 25 alleles each and a range in allele size of 68 bp. Right panels: Gmo4 and Gmo141, both loci with 25 alleles each and a range in allele size of 138 bp. (a) and (a') $(\delta\mu)^2$, (b) and (b') D_{SW} , (c) and (c') R_{ST} , and (d) and (d') F_{ST} . Error bars are 95% confidence intervals obtained as the 2.5th and 97.5th percentile of the empirical distribution of 1000 trials.



and 6). Sampling variances for Rogers' (1972) distance (D_R), Cavalli-Sforza and Edwards' (1967) chord distance (D_C), allele sharing distance (Bowcock et al. 1994), and ASD were largely independent of sample size and number of loci and were always large (Fig. 2), indicating that these four measures perform poorly when applied to microsatellite data (see also Goldstein et al. 1995b for discussion on variance for ASD and Bentzen et al. 1996 and McConnell et al. 1997, for examples).

For $(\delta\mu)^2$, D_{SW} , R_{ST} , and F_{ST} , sampling variances decreased rapidly with increasing sample size and with increasing number of loci. Sampling variances were relatively small for sample sizes ranging between 50 and 100 individuals or larger. For these four measures, the decrease in sampling variance for samples larger than about 100 individuals was generally minor, suggesting that further increases in sample size beyond this range are unlikely to result in increased precision.

Finally, the sampling variance for D_A (Nei et al. 1983) decreased with sample size, but this decrease was not as pronounced as for $(\delta\mu)^2$ or D_{SW} , and what may be more important, within the range of number of loci examined, the sampling variance of D_A did not decrease with increasing number of loci (Figs. 2c vs. 2c'). These results suggest that D_A is less precise than $(\delta\mu)^2$ or D_{SW} for assessing demographic structure using microsatellites, a result that contrasts with Takezaki and Nei (1996), who recently concluded using computer simulation that both D_C (Cavalli-Sforza and Edwards 1967) and D_A (Nei et al. 1983) were superior to $(\delta\mu)^2$ and D_{SW} because they were more likely to generate the correct phylogenetic tree topology. I have shown here that when the primary concern is assessing whether or not there is indeed demographic structure, rather than estimating a tree branch length, D_C and D_A are not the most useful measures with microsatellite DNA because of their large sampling variances and (or) their sample size related bias.

Shriver et al. (1995) examined the effect of sample size and number of loci on the sampling variance of D_{SW} and suggested that sample sizes greater than 25 individuals do not have an appreciable effect on the variance of D_{SW} , particularly when a relatively large number of loci is used. Shriver et al. (1995), however, did not examine the effect of sample size on bias. I have shown here that, regardless of number of loci examined, sample sizes of no less than 50 individuals are probably necessary to minimize bias in the estimation of D_{SW} .

Sample sizes often differ between or among populations. I therefore examined the effect of samples of widely differing sizes on the sampling variance of pairwise estimates. An important finding of this particular analysis was that large differences in sample sizes can lead to widely differing estimates of R_{ST} in pairwise comparisons when R_{ST} is calculated as a ratio of variances, even when all samples are relatively large (Fig. 2h'). This problem can be overcome by estimating R_{ST} following a conventional analysis of variance (ANOVA) approach as suggested by various authors (Kimmel et al. 1996; Michalakis and Excoffier 1996; Goodman 1997). I have shown here that when effective average sample sizes are relatively small ($N < 50$), sampling variances for R_{ST} are relatively large when sample sizes differ, even when estimated within the analysis of variance framework (Fig. 2h'). Thus, it appears that, unless effective average sample sizes are relatively large (i.e., $N_e > 50$), precise estimates of R_{ST} require approximately equal sample sizes. Goodman (1997) also discussed the effect of differential variance between loci as a potential source of inaccurate results when estimating R_{ST} and suggested that this problem can be addressed using a normal

standardization in which allele sizes are expressed in terms of standard deviations from the mean. With the data used in this study the decrease in confidence intervals for R_{ST} with such standardization was negligible at any but the very small sample sizes ($N_e \leq 20$; data not shown). Differing sample sizes had no appreciable effect on any of the other measures, including F_{ST} , suggesting that when there are large differences in sample size and the effective average sample size is small, F_{ST} may be the preferred measure to estimate population structure. However, F_{ST} was not designed with the stepwise mutational model in mind, which is presumed to prevail at microsatellites loci (Shriver et al. 1993; Valdes et al. 1993; Weber and Wong 1993; Di Rienzo et al. 1994; Goldstein et al. 1995a; Slatkin 1995), and thus, F_{ST} does not consider distance among alleles, just whether or not they are equal. Slatkin (1995) compared the performance of R_{ST} and F_{ST} using computer simulation and found that estimates of F_{ST} showed too much genetic similarity when the evolutionary time since population divergence was long relative to the ancestral population size, whereas R_{ST} estimates showed little or no bias (Slatkin 1995). The performance of F_{ST} improved when the time since the splitting of the ancestral population relative to its size was short, because in this case, genetic drift is the predominant process creating local differentiation, and mutation plays a relatively minor role (Slatkin 1995).

Cod microsatellites exhibit very high variability relative to comparable dinucleotide microsatellites in most other species (Brooker et al. 1994; see also O'Reilly and Wright 1995). The average number of alleles observed per locus in the six microsatellites surveyed in the entire larval aggregation was 48 with a maximum of 61 (Gmo4) and a minimum of 29 (Gmo2). Mammalian species, by contrast, have fewer than 20 alleles per locus (chimpanzees, 4–17 (Morin et al. 1994); bears, 8–19 (Paetkau and Strobeck 1994; Paetkau et al. 1995)).

To examine how expected values and sampling variances were affected by allele number and size range, I binned alleles at four of the available loci in two different ways (see Figs. 3–6). In the first case, I compared loci that differed in the number of alleles by a factor of three but were similar in size range. In the second case, I compared loci that shared the same number of alleles but differed markedly in size range. The bias of $(\delta\mu)^2$ is dependent on the variance in allele size and is expected to be independent of the number of alleles. However, both the number of alleles and the distance between alleles are positively correlated under the stepwise mutation model. I manipulated the maximum distance between alleles, and this distance or range in allele size is only one of several factors affecting the variance. Other factors that contribute to variance in allele size are the number and distribution of alleles within a given range of sizes and the shape of the allele frequency distribution.

Factors that I did not address in the present study and that remain to be explored are the effect of changes in allele frequency distribution that occur as a result of the genetic sampling process (e.g., Slatkin and Barton 1989) and in the geographic pattern and degree of population subdivision. Allele frequency distributions in the larval cod data used in the present study appear typical for microsatellites, with most loci showing multimodal and some loci showing slightly skewed allele frequency distributions (Figs. 1 and 3). The performance of some of the measures examined here may change with distributions that are more highly skewed or otherwise depart more markedly from normality.

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