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Assessing Genetic Diversity in Clonal Organisms: Low Diversity or Low Resolution? Combining Power and Cost Efficiency in Selecting Markers

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The increasing use of molecular tools to study populations of clonal organisms leads us to question whether the low polymorphism found in many studies reflects limited genetic diversity in populations or the limitations of the markers used. Here we used microsatellite datasets for two sea grass species to provide a combinatory statistic, combined with a likelihood approach to estimate the probability of identical multilocus genotypes (MLGs) to be shared by distinct individuals, in order to ascertain the efficiency of the markers used and to optimize cost-efficiently the choice of markers to use for deriving unbiased estimates of genetic diversity. These results strongly indicate that conclusions from studies on clonal organisms derived using markers showing low polymorphism, including microsatellites, should be reassessed using appropriate polymorphic markers.

The development of molecular techniques over the last decade has provided new tools to examine genetic variability within and among populations (Avise 1989, 1994; Parker et al. 1998; Sunnucks 2000). However, the efficient use of new molecular techniques often lags behind their accelerating development (Parker et al. 1998). In a variety of cases, conclusions previously drawn from the first markers used, commonly allozymes or random amplified polymorphic DNA (RAPD), have been significantly revised in light of results obtained using new markers, either due to evidence for selection on some markers, or to very low levels of variability limiting their ability to estimate population parameters (Beaumont and Pether 1996; Charlesworth 1998; Lemaire et al. 2000; Parker et al. 1998; Pogson et al. 1995).

Limited marker resolution becomes even more critical when studying clonal organisms for which multilocus genotypes are the only way to discriminate genetically distinct individuals, such as plants or bacteria (Hagen and Hamrick 1996; Hamrick and Godt 1989). Aquatic plants, many of which are highly clonal, are particularly prone to erroneous inferences on the genetic structure of their populations derived from the use of low-power markers. Indeed, initial evidence based on allozymes indicated widespread genetic monomorphism in submerged plants (Barrett et al. 1993), suggesting a much larger role for clonal than for sexual propagation. However, subsequent research using more powerful markers provided evidence of a wider range of clonal diversity, from apparently monoclonal meadows (Waycott et al. 1996) to multiclonal and highly genetically diverse ones (Alberte et al. 1994; Laushman 1993; Reusch et al. 2000; Waycott 1995).

Very low levels of genetic variability have been reported for the Mediterranean sea grass *Posidonia oceanica*, inferred to be highly clonal, both when using allozymes (Capiomont et al. 1996) and RAPD markers (Procaccini et al. 1996; Procaccini and Mazzella 1996). The recent application of new RAPD primers (Jover et al. 2003) and of tri- and heptanucleotide microsatellites (Procaccini and Waycott 1998) revealed higher clonal diversity (Table 1 and Figure 1), suggesting that previous reports of low genetic variability were largely derived from the limited power of allozymes and of the first RAPD markers that were used. Yet the level of genetic variability revealed by those microsatellites remained low, still suggesting a predominance of clonal growth in the maintenance of natural populations (Procaccini et al. 2001). Recently developed dinucleotide microsatellites suggested a much higher level of clonal diversity in the *P. oceanica* meadow used to test for polymorphism (Alberto et al. 2003a). The increasing revelation of the genetic diversity of clonal organisms, as new tools are introduced, questions the extent to which inferences derived from any one marker type provide

Table 1. Sampling methods applied in the studies of population genetics of *P. oceanica* using distinct molecular markers

Markers	SD	N	md	Md	SA	L(p)/P	A	References
Allozymes	H	4–51	—	—	5000	8(2)	2	Capiomont et al. (1996)
RAPD 1	Lr	16	10	40	—	65(1)/11	1	Procaccini et al. (1996)
RAPD 2	H	9–15	5–10	—	—	28(26)/2	2	Jover et al. (2003)
M_{tc}	Lr	20–47	5–8	145–232	—	5	4	Procaccini et al. (2001)
M_d (popul. 1–4, 8)	R	38–50	0.5–1.5	67–79	1600	8	12.5	Present study
(popul. 5–7)	Lr	29–40	5–8	145–232	—			

Details are given as to the markers used (M_{tc}: tri- and heptanucleotide microsatellites, M_d: dinucleotide microsatellites), the sampling design (SD; Lr: linear transect with equally spaced sampling points; R: random coordinates in 80 m × 20 m; H: haphazard sampling), sample size (*N*), the approximate minimum and maximum distances (md, Md, respectively) in meters between sampled shoots, as well as the area sampled when nonlinear sampling was used (SA, in m^2), the number of loci (L), and when distinct, the number of polymorphic loci (p) analyzed, or the number of RAPD primers used (P), the average number of alleles per polymorphic loci (A), and the corresponding references.

reliable accounts of the genetic diversity of these populations or reflect the limitations of these markers (Reusch 2001).

Here we propose a combined approach using both the exploration of all marker combinations and the likelihood probability of identical multilocus genotypes (MLGs) to be shared by distinct individuals to ascertain a priori the dependence of the estimates of genetic diversity of clonal organisms on the number and efficiency of the markers used. This approach can also be used to optimize, in terms of cost-efficiency, the choice of markers to derive unbiased estimates of genetic diversity of the clonal organism studied. We demonstrate this approach using microsatellite markers for two clonal sea grass species, *P. oceanica* and *Cymodocea nodosa* (Alberto et al. 2003a,b).

Materials and Methods

For *P. oceanica*, approximately 40 shoots were collected from each of eight localities (Table 2). A portion of each shoot was desiccated and preserved in silica crystals. For *C. nodosa*, 38 shoots were collected in patches in Alfacs Bay (northern Spain). A sample of 45 seedlings collected in Cadiz Bay was used as a control for marker power, since no clonal replicates are expected in seedlings.

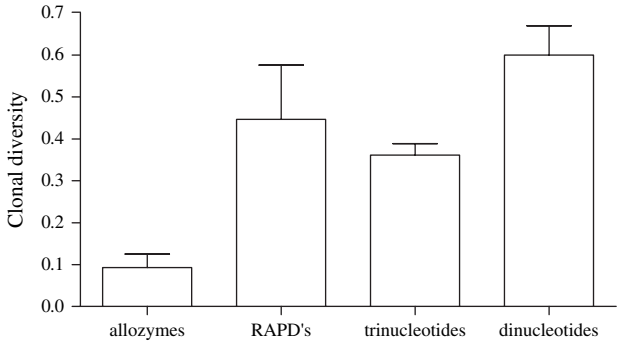


Figure 1. Estimated levels of clonal diversity ($R = G - 1/N - 1$), where G is the number of genotypes and N is the number of samples analyzed, reported in *P. oceanica* meadows with allozymes (A), RAPD (R), tri- and heptanucleotide microsatellites (T), and dinucleotide microsatellites (D).

Genomic DNA was isolated following the CTAB method (Doyle and Doyle 1988). Samples from eight meadows of *P. oceanica* were fully genotyped for four trinucleotides, one 7-nucleotide (Procaccini and Waycott 1998), and eight dinucleotides (Alberto et al. 2003a) microsatellites. The samples of *C. nodosa* were genotyped for 12 dinucleotide microsatellites, as described in Alberto et al. (2003b).

The clonal or genotype diversity is often estimated as $P_d = G/N$, where G is the number of distinct genotypes identified and N is the number of shoots analyzed. However, for a monoclonal stand (i.e., with a single genotype), this index would indicate a different value of genotypic diversity depending on the sample size, and thus we chose to modify it slightly by using $R = G - 1/N - 1$, which will always be zero for a single clone stand and one for maximal genotypic diversity, when every sampled unit is a new genet (Dorken and Eckert 2001). The index maintains the same order of magnitude and can still be approximately compared with the classical P_d values reported in the literature, although care must still be taken when comparing values from studies using different sampling designs.

Genotypic diversity as revealed by any possible combinations of any number of the available markers was then computed in order to select the most parsimonious marker combination allowing efficient discrimination of genets. Submatrices were produced from the complete genotype matrix by selecting all combinations C_l^L of l from the L available loci (with $1 \leq l \leq L$ and $L = 5$ for tri- and heptanucleotides, and $L = 8$ for dinucleotides). For each rank of l, the average genotypic diversity R was computed, as well as the standard error, and the combination of l loci revealing more distinct genotypes was retained. This exercise was performed using a routine written in C (Gencount, available from F. Alberto upon request) on each population and both sets of markers (tri- + heptanucleotides, and dinucleotides). The curves describing the dependence of the average R (± SE) on l were drawn and compared. The minimal combination of l markers allowing the discrimination of the maximum MLGs with a satisfying likelihood probability was retained and compared among populations in order to find a minimal consensus combination allowing the discrimination of all MLGs in any population.

In addition, and to test whether all of the samples with identical genotypes belong to the same genet, we used the round-robin method (Parks and Werth 1993) to estimate allelic

Table 2. Sampling details for *P. oceanica* populations used in this study

Geographic zone (from west to east)	Sampling location	SD	N_s	N_g	5triR	5diR	R
Northern Spain	Port Lligat	R	40	13	0.17	0.24	0.31
Southern Spain	Las Rotes	R	50	34	0.23	0.58	0.67
Balearic islands (Formentera)	Illetes	R	36	23	0.22	0.54	0.63
Balearic islands (Formentera)	Es Calo de Oli	R	40	15	0.23	0.34	0.36
Italy (Sardegna)	Tavolara	Lr	40	20	0.20	0.41	0.49
Italy (southeastern)	Otranto	Lr	29	24	0.21	0.70	0.82
Malta	Malta	Lr	39	33	0.14	0.78	0.84
Cyprus	Paphos	R	38	26	0.21	0.58	0.68

Sampling location, sample size (N_s), sampling design (SD; Lr: linear transect with equally spaced sampling points; R: random coordinates in 80 m × 20 m). number of genotypes (N_g), average clonal diversity with five di- and tri- + heptanucleotide microsatellites (5 × R) microsatellites, and with all the eight dinucleotides (R).

frequencies and genotype probabilities in each population. This subsampling approach avoids overestimation of rare allele frequencies by estimating the allelic frequencies for each locus on the basis of a sample pool composed of all the genotypes distinguished on the basis of all the loci, except the one being estimated. This procedure is repeated for all loci, and the unique genotype probability is then calculated as follows:

$$\mathrm{p}_{gen} = \prod_{i=1}^{l} (f_i) 2^b,$$

where l is the number of loci, f_i is the frequency in the population of each allele (two per locus) at the ith locus, and b is the number of heterozygous loci. When the same genotype is detected more than once, the probability of these being derived from distinct reproductive events (i.e., different genets) can be estimated by the binomial expression

$$\mathrm{p}_{sex} = \sum_{x=n}^{N} \frac{N!}{x!(N-x)!} \times [p_{gen}]^x \times [1 - p_{gen}]^{N-x},$$

where N is the number of sampling units and n is the number of separated fragments with identical genotype to a previously encountered ramet (Parks and Werth 1993; Stenberg et al. 2003; Tibayrenc et al. 1990). In order to test whether all replicates of a MLG belong to the same genet, significance was considered for $\mathrm{P}_{\mathrm{sex}}$ from the first re-encounter (n = 1).

In order to evaluate the importance of somatic mutations, the frequency distribution of the number of different alleles between all pairs of MLGs was plotted for each population.

Results

The analysis of clonal diversity performed on eight *P. oceanica* populations with four trinucleotide and one heptanucleotide nuclear microsatellites (Figure 2a) showed low levels of diversity ranging from 0.14 (Malta) to 0.23 (Formentera Illetas). Much higher diversity was revealed when using the same number (five) of dinucleotides (Figure 2b); the level of diversity ranged from 0.24 (Port Lligat) to 0.78 (Malta). With all eight dinucleotide loci, the variability was between 0.31 (Port Lligat) and 0.84 (Malta). In the same way, the number of alleles per locus was 3 with the septanucleotides, approximately 4 with trinucleotides, and 12.5 with dinucleotides.

For *C. nodosa*, all seedlings had different MLGs (Figure 2c) as expected, whereas lower clonal diversity of approximately 0.44 was observed for the northern Spain population. However, both samples reached the maximum clonal diversity with a minimum combination of four markers.

In all the *P. oceanica* samples, all identical genotypes identified with dinucleotides were estimated to have a probability of less than .01 of having originated from two or more distinct events of sexual reproduction (i.e., distinct genets), except for three genotype pairs from Paphos and Port Lligat (where $.01 < P_{\mathrm{sex}} < .05$), whereas this was not the case for tri- and heptanucleotide microsatellites. In all populations, some MLGs defined with these last markers showed a higher probability ($P_{\mathrm{sex}} > .05$), thus indicating that several distinct individuals may be included in the same MLG groups (data not shown). Indeed, this was confirmed by the discrimination within those groups of several individuals bearing distinct MLGs when using dinucleotide microsatellites. In the same way, the relationship between the number of tri- and heptanucleotide microsatellites and the apparent genetic diversity (Figure 2a) indicated that even using the full set underestimates the genetic diversity of *P. oceanica* populations. In contrast, we observed an asymptotic relationship between the number of dinucleotide markers used and the clonal diversity revealed by these for most populations of both *P. oceanica* and *C. nodosa*, which reached unity for the *C. nodosa* seedlings, as expected (Figure 2). This relationship indicates that it is possible to select one or several subsets of markers yielding accurate estimates of clonal diversity for these species. The optimal combination of markers, leading both to an asymptotic shape and to low P_{sex} values ($<.05$), was found to be, for *P. oceanica*, a set of seven dinucleotides, and for *C. nodosa*, a set of six dinucleotides.

Finally, the frequency distribution of the number of different alleles between all MLG pairs showed unimodal distributions in all populations, with no apparent trend toward higher frequency or extra peak at very low values, as might be expected if somatic mutation was a common event (see the appendix). The seed sample of *C. nodosa* can be considered as a control distribution in which no somatic mutation should be expected. Slightly more low values can be observed in *P. oceanica* than in *C. nodosa*, which may be attributed to their distinct mating system, *P. oceanica* being monoecious and able to self-fertilize, whereas *C. nodosa* is dioecious.

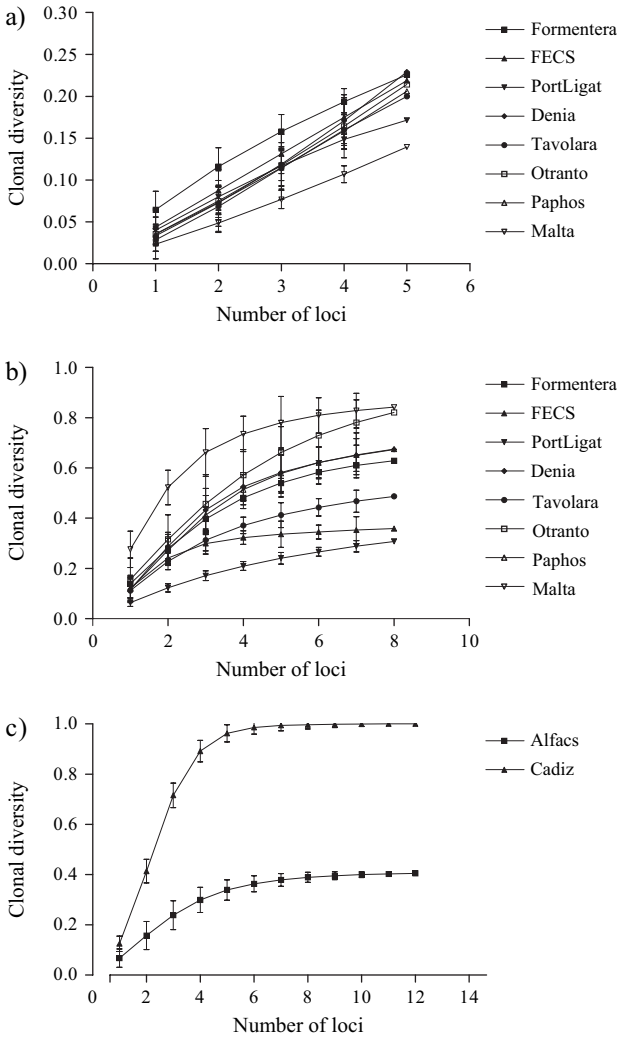


Figure 2. Curves describing the genotypic resolution of microsatellites with (a) tri- and heptanucleotide and (b) dinucleotide motifs in *P. oceanica*, and in (c) *C. nodosa*, based on analysis of all possible combinations C_n^l of n loci ($n = 1,\ldots,l$; $l =$ number of loci available), giving the average clonal diversity R (±SE) for each n. Clonal diversity estimated by $R = G - 1/N - 1$, where G is the number of genotypes and N is the sample size.

Discussion

Very different conclusions can be reached concerning the level of genetic polymorphism, either in terms of alleles or genotypic diversity, depending on the markers used (Figure 1 and Table 2), not only between allozymes (R = 0.14) and RAPD (an average of 0.07 in the first study and 0.49 in the second study), but dinucleotide (0.60) and tri- and heptanucleotide (0.38) microsatellites. One may be concerned by the influence of distinct sampling strategies on this result. However, the area sampled for allozymes studies was larger than for any other studies (Table 1), which then cannot explain the lowest level of genotypic diversity observed with those markers. More important, samples collected from eastern to western Mediterranean localities have common allozyme MLG profiles. As for studies using RAPD or microsatellites, the sampling scheme and average distance between shoots were similar, except in the study with dinucleotide microsatellites, in which the distance between shoots was less (Table 1), which would nevertheless tend to increase the chance to sample shoots from the same clones and would therefore not cause the higher genotypic diversities observed here for these markers. However, in the present study, a very different estimation of R could be derived from analysis of the same number of markers (five) on the same population samples with dinucleotide (0.52) and tri- and heptanucleotide (0.20) microsatellites (Figure 2a,b and Table 2).

Reports of limitations linked to microsatellite markers are more scarce than limitations linked to allozymes or RAPD (Allendorf and Seeb 2000; Beaumont and Nichols 1996; Parker et al. 1998). However, different levels of genetic variability depending on microsatellite motif have been reported in some eukaryote genomes, and the relative mutation rate of dinucleotides versus tri- and heptanucleotides is estimated to be between 1.5 and 2.1 (Anderson et al. 2000; Chakraborty et al. 1997). This is thought to be due to both the differential mutation rate during replication and to a higher rate of recombination and consequent mismatch repair (Chakraborty et al. 1997; Li et al. 2002). The average number of alleles per locus is a linear function of $4N_e\mu$, where N_e is the effective population size and μ is the mutation rate (Kimura 1983). In our study, this number is 2.5 to 3 times higher for dinucleotides than for trinucleotides, instead of 1.5 to 2.1, suggesting a ratio of respective mutation rates (μ_{di}/μ_{tri}) somewhat higher than estimated thus far, although this could be a consequence of variance due to the limited number of microsatellites used. This mutation rate should lead to careful screening for possible bias due to somatic mutations at dinucleotide loci. However, in the present study, the frequency distribution of the pairwise number of allele differences in all populations did not show evidence for a significant bias of genotypic diversity due to somatic mutations. Although slightly more low values can be observed in *P. oceanica* than in *C. nodosa*, this may be due to their distinct mating system, *P. oceanica* being monoecious and able to self-fertilize, whereas *C. nodosa* is dioecious. A higher frequency of related individuals may then be observed in *P. oceanica* meadows. Yet no secondary peak toward very low values was observed, but instead, an unimodal distribution shape appeared to be the rule.

The perils of drawing inferences on the population structure of clonal organisms using markers of insufficient resolution are evident when comparing the results obtained in *P. oceanica* with different microsatellites. In particular, the population sampled in Malta exhibited the lowest number of clones with tri- and heptanucleotides (R = 0.14), suggesting this meadow relies on clonal (versus sexual) reproduction (Table 2 and Figure 2a). However, the population in Malta clearly emerges as one of the most genetically diverse (R = 0.84) when examined with the more powerful dinucleotide microsatellites, suggesting high level of sexual reproduction in this particular meadow (Table 2 and Figure 2b). Hence the use of markers of limited power introduces both quantitative and qualitative errors in assessments of the genetic diversity of populations of clonal organisms, as not only was clonal

diversity underestimated, but the relative ranking of clonal diversity among the populations examined was also in error.

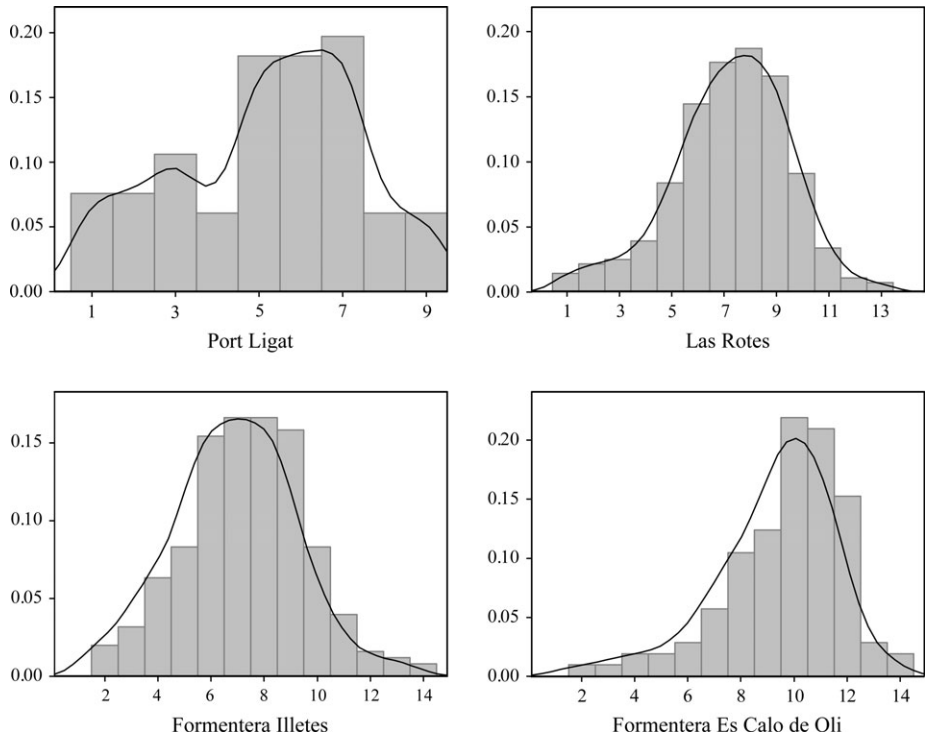
The combined method reported here provides a convenient method for selecting the best combination of microsatellites to obtain accurate descriptions of the genetic diversity of populations of clonal organisms, provided sufficient highly polymorphic markers are available. The results presented confirm the expected asymptotic shape of the relationship between the number of microsatellites used and the genetic diversity they reveal (Figure 2b,c), and demonstrate that failure to observe such asymptotic patterns is indicative of insufficient power (Figure 2a). In addition, all the calculated probabilities for any pair of identical genotypes to be derived from two distinct events of sexual reproduction (P_{sex}) were less than .05, further indicating that unambiguous estimates of clonal diversity of the meadows examined could be derived with the markers used. The usefulness of this combined approach is stressed by the fact that it would have warned of the likelihood of drawing erroneous inferences on the population structure of *P. oceanica* with the use of the tri- or heptanucleotide markers, where no genotypic diversity asymptotic value was reached and identity probabilities were not significant for all groups of MLG. The application of the method to *C. nodosa* seedlings (Figure 2c), for which the relationship between the number of microsatellite markers used and the estimated genetic diversity converged to the expected asymptotic value of one, further confirms that the approach provided here allows assessment of the accuracy of inferences on the genetic diversity of clonal organisms derived using different sets of markers.

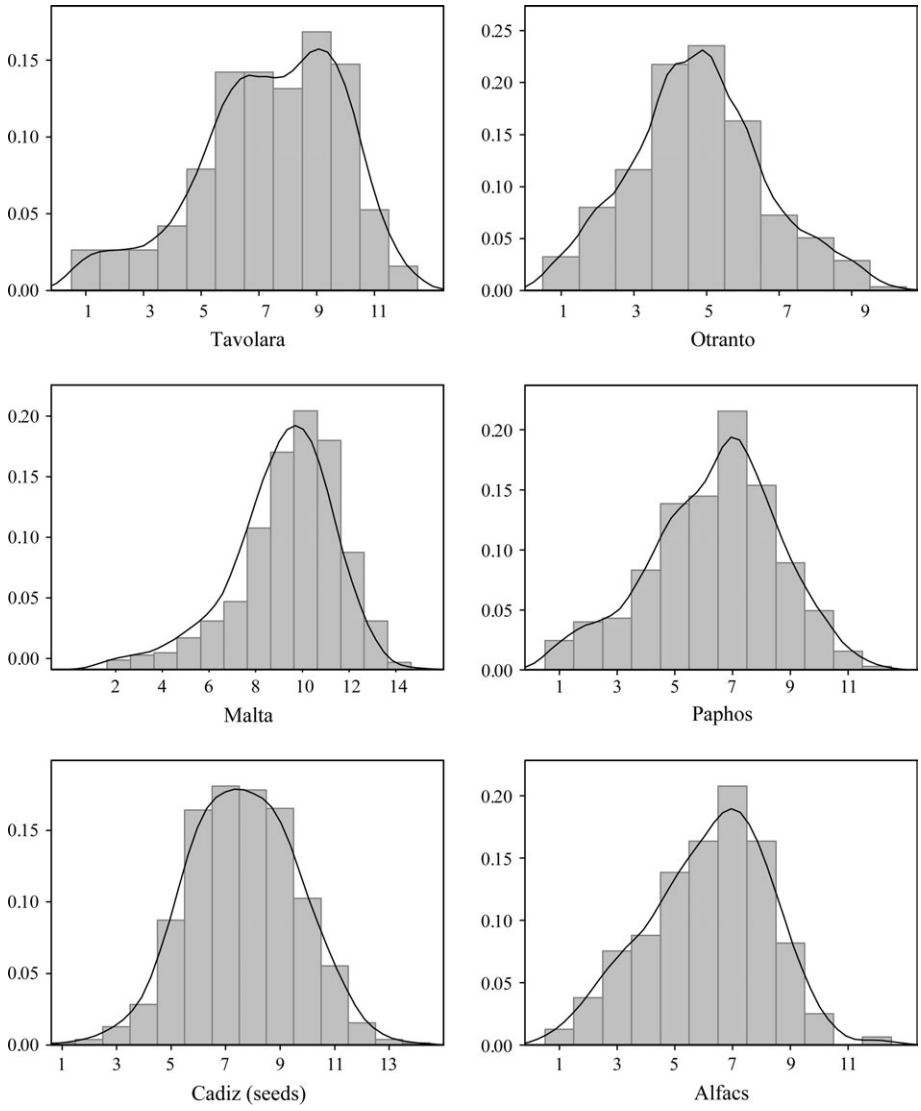
Although genotyping as many distinct loci as possible will ensure the maximal genotype diversity of the samples analyzed, this practice is seldom realistic because of resource limitations, both time and money. Under these circumstances, the procedure can also been used in a pilot study to select the minimum combination of markers delivering accurate estimates of genetic diversity (i.e., asymptotic values and significant P_{sex} values), thereby helping to optimize the cost-efficiency of research efforts in terms of genotyping and in terms of time and money. The most cost-effective combinations include seven and six markers for *P. oceanica* and *C. nodosa*, respectively.

In conclusion, the results presented here demonstrate the risks of delivering quantitatively and qualitatively erroneous inferences on the genetic diversity of populations of clonal organisms when the markers available have insufficient power. These results show that conclusions about low sexual reproduction in populations of clonal species derived with the use of markers showing low polymorphism (i.e., a small number of alleles, or not evenly distributed) need to be reassessed using markers capable of revealing the distinct genotypes of the population. The approach provided here, applicable to any clonal organism, allows the combined assessment of the asymptotic trend of the markers and of the significance of the associated likelihood probability of identity in order to ascertain the detection of all distinct genotypes sampled, and thus to provide accurate estimates of population genetic diversity, while estimating the most cost-effective combination of markers to achieve this goal.

Appendix

Frequency distribution of the pairwise number of allele differences between MLGs in each of eight *P. oceanica* samples and in two *C. nodosa* samples. The *x*-axis represent





the number of allele differences and the *y*-axis is the frequency distribution for each *x* rank.

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