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Assessing population genetic structure of sorghum landraces from North-western Morocco using allozyme and microsatellite markers

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Abstract The level of genetic diversity and the population genetic structure of sorghum landraces from North-western Morocco have been investigated based on direct field-sampling using both allozyme and microsatellite markers. As expected, microsatellite markers showed a much higher degree of polymorphism than allozymes, but relative measures of genetic structure such as Wright's inbreeding coefficient F_{IS} and Nei's coefficient of genetic differentiation G_{ST} were similar for the two sets of markers. Substantial inbreeding was found to occur within fields, which confirms that sorghum is predominantly selfing under cultivation. Most of the genetic diversity in Moroccan landraces occurs within fields (more than 85%), as opposed to among fields or among regions, a result which contrasts to those of studies based on accessions from germplasm collections. It is suggested that individual fields of sorghum constitute valuable units of conservation in the context of in situ conservation practices.

Key words Allozymes · Genetic diversity · Genetic resources · Microsatellites · Sorghum

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Introduction

Molecular markers are recognised as significant tools to orient plant genetic resource conservation management, providing a means to accurately estimate the genetic diversity and genetic structure for species of interest (Hamrick and Godt 1997). They have been used, in the context of the ex situ conservation of domesticated species, to assess the pattern of genetic diversity in large germplasm collections (e.g. Lubbers et al. 1991; Zhang et al. 1992), to suggest priorities in future sampling missions, or to optimise the assembly of core collections (Schoen and Brown 1995). In the context of the in situ conservation of landraces, molecular markers could be useful to facilitate the selection of optimum sites, as well as to monitor ongoing changes in the pattern of diversity in the course of conservation practices (Newbury and Ford-Lloyd 1997). Currently, the pattern of genetic diversity within large germplasm collections is well characterised for most crops (e.g. Morden et al. 1989), but this does not necessarily reflect the extant genetic structure of landraces under cultivation conditions. Several factors could account for discrepancies between genetic-diversity estimates based on direct field samples and on accessions taken from germplasm collections: (1) most studies involve extremely low sample sizes at the accession level (less than ten seeds per accession in most cases: Doebley et al. 1985; Morden et al. 1989); (2) the plant material in germplasm collections is likely to have passed through genetic bottlenecks because of sampling and regeneration procedures; (3) the geographic scale of interest differs between genebank (world-wide scale) and in situ studies (regional and narrow scale). For these reasons, investigations on the pattern of genetic diversity of landraces in situ are urgently needed in order to orient in situ conservation programmes.

Sorghum (*Sorghum bicolor* L.) is one of the major food grains in the world, cultivated mainly in North

America, Africa and Asia, with its original centre of diversity located in Ethiopia (De Wet and Huckabay 1967; Doggett 1988). According to the classification of Harlan and De Wet (1972), five basic races of cultivated sorghum are recognised, with a number of intermediates registered in ten categories. Sorghum is a wind-pollinated annual crop with outcrossing rates of about 0.10–0.15 (Doggett 1988), and hybridisation with spontaneous species seems to occur frequently. Allozyme studies in cultivated sorghum showed that it is strikingly less variable than other cereals like maize or barley, and that the considerable differentiation among accessions follows differences in geographic origin rather than racial classification (e.g. Morden et al. 1989; Ollitrault et al. 1989 b). Most of these conclusions were confirmed from investigations on molecular markers such as RAPDs and nuclear RFLPs (e.g. Tao et al. 1993; Deu et al. 1994; Cui et al. 1995), mitochondrial DNA (Deu et al. 1995) and chloroplast DNA markers (Aldrich and Doebley 1992). However, only genebank accessions from world-wide origins were used in these studies, and the in situ pattern of genetic diversity at a regional scale remains unknown.

In this paper, we investigate the population genetic structure of sorghum landraces from North-western Morocco, based on direct sampling in farmers' fields. In this region, sorghum is mostly cultivated under traditional practices, and used for feeding livestock and poultry, as well as a backup in human nutrition (Kadiri and Ater 1997). A high degree of morphological variation has been observed within Moroccan landraces, which were assigned to the races durra, bicolor, and their intermediates (Kadiri and Ater 1997). In a previous study, we analysed the patterns of morphological and allozyme variation in Moroccan sorghum based on samples from six fields (Djè et al. 1998). We showed that the differentiation among fields was considerably larger for morphological characters than for allozyme markers (respectively 63% and 20% of the total variation expressed among fields), but this sampling scheme did not allow a detailed inference on the pattern of genetic variation. In the present paper, we used a hierarchical sampling design, with 20 fields of sorghum from North-western Morocco sampled within five separate areas, and investigated both allozyme and microsatellite markers. We addressed the two following issues: (1) the partition of genetic diversity at three different levels (individuals, fields, regions); (2) the comparison of population genetic statistics obtained with microsatellites versus allozymes.

Materials and methods

Plant material

A hierarchical sampling design was used with five representative regions from North-western Morocco prospected in August 1996 and four fields chosen within each region (Fig. 1). The prospected areas were situated in the vicinity of Tanger and Tetouan and ranged from low (running along the Mediterranean sea) to moderately high altitude (800 m). In each field, 15 inflorescences from different individuals were harvested at random. One seed per inflorescence was allowed to germinate in a Petri dish in a dark room at 22°C and seedlings were then transferred to the greenhouse and grown for about 1 month.

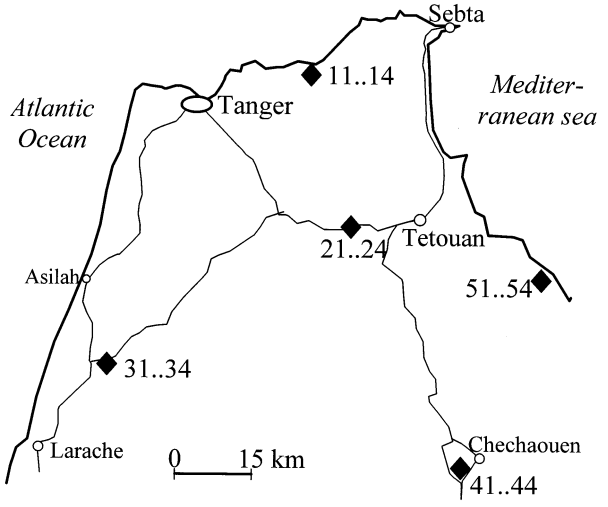

Fig. 1 Locations of sorghum fields sampled from five regions of North-western Morocco. Four fields were sampled per region and denoted as S_{ij} for the ith region and the jth population

Isozyme procedures

Leaves from 4–6 week-old seedlings were harvested and ground on ice in the extraction buffer [Tris HCl 78.68 mM, polyethyleneglycol (PEG) 6000: 1%, polyvinilpyrrolidone (PVP-40): 2%, dithiothreitol (DTT) 2.54 mM, sucrose 146 mM, ascorbic acid 2.02 mM, sodium metabisulfite 20 mM, 2-mercaptoethanol 0.1%]. Extracts were centrifuged for 20 min at 13 000 g and then the supernatant stored at − 75°C. The homogenates were used in vertical 7.5% polyacrylamide gels in a Tris-glycine buffer (pH 8.6) according to Hames and Rickwood (1990). Running conditions were 75 mA for the upper gel (45 min) and 150 mA or 300 V for the lower gel (2 h 30 min) with a Protean II xi Slab Cell. In addition, part of the supernatant was adsorbed on Whatman 3M paper just after centrifugation and conserved at − 75°C for starch-gel electrophoreses. The latter were run on 12% starch (Sigma # S-8501) gels (180 V for 5 h) in a continuous Tris-borate-EDTA pH 8.6 buffer (Wendel and Weeden 1990). All electrophoreses were carried out in a cold chamber at 4°C. Nine enzymes giving clear patterns were employed in routine procedures [AAT (E.C 2.6.1.1), ADH (EC 1.1.1.1), DIA (EC 1.8.1.4), EST (EC 3.1.1.-), GPI (EC 5.3.1.9), PER (EC.1.11.1.7), PGDH (EC 1.1.1.44), PGM (EC 5.4.2.2) and SOD (EC 1.15.1.1)]. Visualisation recipes are those given by Wendel and Weeden (1990). Genetic control of the analysed enzymes is discussed by Ollitrault et al. (1989 a) and Wendel and Weeden (1990).

Microsatellite analysis

Total DNA was extracted from 100 to 150 mg of leaves following a CTAB procedure (Doyle and Doyle 1990) that yielded from 8 to

15 μg of total DNA. A dilution (1/100) of these total DNA extracts was consecutively used in PCR reactions. Primer pairs of three microsatellite loci that showed polymorphism on a world-wide sample of sorghum were taken from Brown et al. (1996): *sb4-22*, with the composite motif ACGAC/AG; *sb5-236*, an AG repeat; and *sb6-325*, an AAG repeat. PCR conditions have been optimised for each primer pair by adapting the annealing temperature (Tm) and/or the $MgCl_2$ concentration in the reaction mix. The PCR reactions were performed from approximately 15 ng of a total DNA extract in 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, varying concentrations of $MgCl_2$ and 0.5 unit per reaction of Goldstar™ DNA polymerase (Eurogentec) for a total reaction volume of 25 μl. PCR cycling conditions were: 2 min initial denaturation at 95°C, 30 cycles of (30 s at 95°C, 45 s at Tm, 1 min elongation at 72°C), followed by a final elongation of 10 min at 72°C. All PCR reactions were performed on a Gene-E thermocycler (Techne). The results of these amplifications were analysed on 8% non-denaturing polyacrylamide gels (16-cm length) run vertically (either 92 V for 15 h for DNA fragments shorter than 200 bp, or 150 V for 16 h for longer amplification products). Amplified fragments were visualised and sized using the Gel Doc 1000™ image analysis system (Biorad) after ethidium bromide staining.

Data analysis

Genetic analyses were performed on allozyme and microsatellite genotypic data using the computer program GEN-SURVEY (Vekemans and Lefèbvre 1997). The level of inbreeding was measured using Wright's inbreeding coefficients F_{IS} (at the population level) and F_{IT} (at the whole data-set level) according to Weir and Cockerham (1984). The following measures of genetic variation within fields were calculated: mean number of alleles per locus (A); mean number of alleles per polymorphic locus (A_p); proportion of polymorphic loci at the 5% level (P); observed heterozygosity (H_o); gene diversity (H_e). Genetic structure at the field and region levels was investigated using Nei's analysis of genetic diversity (Nei 1973). Departure from Hardy-Weinberg genotypic proportions within fields was tested using exact tests with the computer program Genepop version 3.1 (Raymond and Rousset 1995). A UPGMA cluster analysis was performed on a matrix of short-term genetic distances between fields based on the co-ancestry coefficient according to Reynolds et al. (1983).

Results

Number of alleles at allozyme and microsatellite loci

We scored 14 allozyme loci and observed a total of 20 alleles. Four loci were polymorphic in the overall sample with two alleles detected (*GPI-1*, *GPI-2*, *DIA-2* and *SOD-2*), whereas one locus (*DIA-3*) showed three distinct alleles. The remaining nine loci were monomorphic: *AAT*, *ADH-1*, *ADH-2*, *DIA-1*, *EST-1*, *EST-2*, *PGDH*, *PGM* and *SOD-1*.

The three microsatellite loci *Sb4-22*, *Sb5-236* and *Sb6-325* were polymorphic with, respectively, 7, 10, and 16 alleles scored in the overall sample. For the highly polymorphic *Sb6-325*, a trinucleotide repeat, we were able to detect all 16 possible alleles in the size interval of 106–151 bp.

Genotypic structure and departure from Hardy–Weinberg proportions

Over the total sample of North-western Moroccan sorghum, we observed a mean F_{IT} value of 0.797 (SD = 0.243) for allozymes and 0.675 (SD = 0.069) for microsatellites, meaning that sorghum landraces are highly inbred overall. The average F_{IS} is 0.787 (SD = 0.247) for allozymes and 0.635 (SD = 0.128) for microsatellites, suggesting that substantial inbreeding also occurs within each field, an indication of the high degree of self-fertilisation of sorghum under cultivation. Tests of departure from Hardy–Weinberg genotypic proportions show a highly significant disequilibrium within fields for the two sets of markers. For allozyme markers, all fields are in disequilibrium at all polymorphic loci, except some cases with *GPI-2* for which the common allele is close to fixation. For microsatellites, 54 out of 60 tests indicate a significant departure from Hardy–Weinberg genotypic proportions.

Genetic variation within fields

Measures of genetic variation within fields have been computed separately for allozyme and microsatellite markers (Table 1). As expected, all statistics are much higher for microsatellites than for allozymes. With a mean of only 1.2 alleles per locus and 1.2% of heterozygous individuals at a given locus, allozymes are poorly informative as compared to microsatellites which show 6.0 alleles per locus and 27.2% heterozygotes on average. The level of within-field gene diversity (H_e) varies greatly among fields when estimated from allozymes (CV = 40.8%), whereas the variation is much less with microsatellites (CV = 6.8%). Inspection of Table 1 also shows that variation among fields in values of gene diversity occurs similarly within each region, so that no substantial differences in diversity are detected among regions, with the sole exception of region 4 which shows less diversity with allozymes but not with microsatellites.

Population genetic structure

The description of the partition of genetic diversity among fields is given in Table 2. Total gene diversity (H_T) is higher for microsatellite markers (0.837) than for allozymes (0.164). Within-field gene diversity (H_S) is consistently higher than diversity among fields (D_{ST}), which represents only about 10% of the total gene diversity ($G_{ST} = 0.074$ for allozymes and 0.143 for microsatellites). The diversity among fields (D_{ST}) can be further divided into gene diversity among fields within regions (D_{SR}), which was found to represent 3.7% and 9.2% of the total diversity for allozymes and microsatellites respectively, and gene diversity among regions

Table 1 Genetic variation within fields of sorghum belonging to five regions from North-western Morocco for allozyme and microsatellite markers

Field	Allozymes					Microsatellites		
	P[a]	A[b]	A_p[c]	H_o[d]	H_e[e]	A_p[c]	H_o[d]	H_e[e]
Region 1								
11	14.3	1.1	2.0	0.009	0.024	7.0	0.458	0.747
12	7.1	1.1	2.0	0.005	0.028	6.7	0.314	0.792
13	21.4	1.2	2.0	0.033	0.069	6.7	0.210	0.740
14	21.4	1.2	2.0	0.005	0.031	6.7	0.159	0.721
Region 2								
21	14.3	1.2	2.0	0.005	0.029	5.7	0.365	0.717
22	28.6	1.3	2.0	0.019	0.084	5.0	0.351	0.689
23	28.6	1.3	2.0	0.020	0.053	5.3	0.200	0.725
24	28.6	1.3	2.0	0.014	0.061	5.7	0.227	0.768
Region 3								
31	35.7	1.4	2.2	0.018	0.095	5.3	0.324	0.607
32	21.4	1.2	2.0	0.019	0.067	5.7	0.306	0.719
33	14.3	1.1	2.0	0.009	0.025	5.3	0.187	0.694
34	28.6	1.3	2.0	0.009	0.058	7.7	0.267	0.759
Region 4								
41	28.6	1.3	2.0	0.010	0.037	5.7	0.092	0.738
42	14.3	1.2	2.0	0.005	0.032	6.0	0.355	0.710
43	14.3	1.2	2.0	0.005	0.044	5.0	0.205	0.681
44	21.4	1.2	2.0	0.014	0.045	4.7	0.133	0.606
Region 5								
51	35.7	1.4	2.0	0.010	0.070	5.3	0.200	0.684
52	28.6	1.3	2.0	0.010	0.069	6.0	0.346	0.775
53	28.6	1.3	2.0	0.014	0.080	7.3	0.367	0.758
54	35.7	1.4	2.0	0.014	0.076	6.7	0.378	0.744
Mean	23.57	1.25	2.01	0.012	0.054	5.97	0.272	0.719
SD[f]	8.39	0.08	0.04	0.007	0.022	0.84	0.097	0.049
CV[g] (%)	35.6	6.4	2.0	56.8	40.8	14.1	35.8	6.8

[a] Proportion of polymorphic loci at the 5% level
[b] Mean number of alleles per locus
[c] Mean number of alleles per polymorphic locus
[d] Average observed heterozygosity
[e] Gene diversity
[f] Standard deviation
[g] Coefficient of variation

Table 2 Analysis of genetic diversity in sorghum landraces from North-western Morocco at allozyme and microsatellite loci

Allozymes					Microsatellites				
Locus	Ht[a]	Hs[b]	Dst[c]	Gst[d]	Locus	Ht[a]	Hs[b]	Dst[c]	Gst[d]
SOD-2	0.142	0.129	0.013	0.091	*sb4-22*	0.802	0.637	0.165	0.206
DIA-2	0.304	0.269	0.035	0.114	*sb5-236*	0.838	0.723	0.115	0.137
DIA-3	0.065	0.061	0.004	0.057	*sb6-325*	0.870	0.797	0.074	0.085
GPI-1	0.114	0.106	0.008	0.071					
GPI-2	0.196	0.189	0.008	0.038					
Mean	0.164	0.151	0.013	0.074	Mean	0.837	0.719	0.118	0.143
SD	0.091	0.081	0.012	0.029	SD	0.034	0.080	0.046	0.061

[a] Total gene diversity
[b] Mean gene diversity within fields
[c] Mean gene diversity among fields
[d] Proportion of among-fields differentiation

(D_{RT}), accounting for 3.7% and 5.0% of the total diversity, respectively. Hence little differentiation is found among fields and especially among regions, a surprising result given the observed levels of inbreeding and geographic separation. UPGMA cluster analyses performed separately on genetic-distance matrices from allozyme and microsatellite markers are illustrated in Fig. 2. Fields belonging to the same region appear

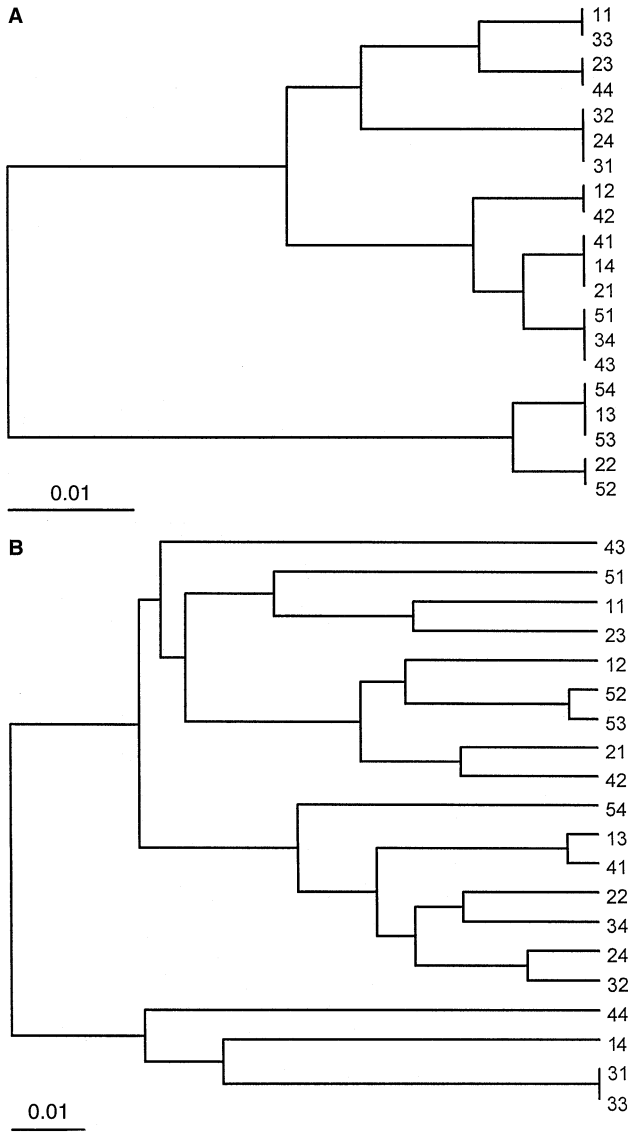

Fig. 2 Dendrogram of genetic differentiation among sorghum fields sampled from five regions of North-western Morocco based on UPGMA cluster analysis of a matrix of pairwise co-ancestry distances based on **a** allozyme data and **b** microsatellite data

widely scattered within each tree. Moreover, there is no concordance between the two trees, i.e. genetic distances based on allozymes and microsatellites are not correlated. For instance, fields 13 and 41 which are very similar for microsatellites belong to very distant clusters for allozymes.

Discussion

Mating system of sorghum under cultivation

Values of inbreeding coefficients are rarely given in the literature on sorghum, but Cui et al. (1995) reported a value of $F_{IS} = 0.79$ obtained from nuclear RFLP markers, which is consistent with a predominantly selfing mating system. This value is close to our estimate based on allozyme and microsatellite markers, $F_{IS} \approx 0.7$, which would be expected under the mixed mating-system model for a selfing rate of $s = 2F_{IS}/(1 + F_{IS}) = 0.82$. Similar estimates of the selfing rate of sorghum have been reported using direct methods based on progeny assays by Ellstrand and Foster (1983) in experimental fields (mean $s = 0.70$), and by Ollitrault et al. (1997) in a study of landraces from Burkina-Faso belonging to the guinea race (mean $s = 0.81$).

Level of genetic variation within fields

For allozymes, the average gene diversity ($H_e = 0.054$) and the number of alleles per polymorphic locus ($A_p = 2.01$) observed in this study are lower than corresponding values reported by Hamrick and Godt (1997) for crop species in general ($H_e = 0.116$, $A_p = 2.67$) or for selfing species ($H_e = 0.092$). Thus sorghum appears to exhibit somewhat less genetic variation in allozymes than other cereals crops, as already noted by Morden et al. (1989). Ollitrault et al. (1997) reported a mean gene diversity of $H_e = 0.075$ for landraces of sorghum from Burkina-Faso, based on grain stocks from ORSTOM/IBPGR sampling missions in 1982. However, this estimate was based on data from ten polymorphic loci, whereas seven monomorphic loci were discarded. When adjusting for monomorphic loci, this gives a mean $H_e = 0.044$, a value very close to our estimate for Moroccan landraces. In contrast, the mean value of H_e reported here is much higher than that obtained within 83 accessions from a world-wide collection of sorghum by Morden et al. (1989), $H_e = 0.008$, which is closer to the value we obtained for the observed heterozygosity ($H_o = 0.012$). This is an important point showing that individual accessions of sorghum are merely copies of the same genotype, whereas fields of sorghum from North-western Morocco contain a large number of distinct, albeit predominantly homozygous, genotypes maintained by self-fertilisation. This finding has implications for devising optimal sampling strategies for conservation purposes, as it stresses the need for seed collection on a large number of individuals per field. Also, this suggests that surveys with genetic markers made on plant material from germplasm collections may greatly underestimate the amount of genetic variation present within fields under cultivation conditions, knowledge of which is of considerable interest for the elaboration of in situ conservation programmes (Newbury and Ford-Lloyd 1997).

Genetic variation among fields

Hamrick and Godt (1997) reported that the average proportion of gene diversity among populations of

crop species (G_{ST}) based on allozyme data is 0.34, whereas a value of 0.59 was observed when considering only selfing crop species. Morden et al. (1989) reported a $G_{ST} = 0.82$ among accessions within a given race of cultivated sorghum, but a $G_{ST} = 0.91$ among accessions in the whole germplasm collection. Ollitrault et al. (1997) reported a $G_{ST} = 0.71$ from a collection of landraces from Burkina-Faso. Our estimate of G_{ST} is much lower than that reported by all previous studies ($G_{ST} \approx 0.1$). Low values of G_{ST} could be caused either by the occurrence of frequent gene flow among fields, for instance as a consequence of seed exchanges among farmers, or by a restriction of the intensity of genetic drift due to a high effective population size and the recent history of sorghum cultivation in Morocco. Based on farmers' interviews (Ater, unpublished), it appears that seeds are most generally taken from the previous harvest, and in cases of accidental loss, new seeds are obtained from neighbouring farmers or local markets. However, our results do not indicate any substantial geographic effect influencing population structure. As far as in situ conservation practices are concerned, our results suggest that individual fields constitute valuable units of conservation, as most neutral genetic variation apparently occurs within fields. Such a conclusion could not have been reached based on information from germplasm material. In previous work, we have shown that differentiation among fields of sorghum was much higher for agromorphological traits than for allozyme markers (Djè et al. 1998). Hence, the choice of fields for in situ conservation should be based mostly on differences in agromorphological characters, because genetic markers do not discriminate among potentially useful groups of Moroccan landraces.

Comparison between allozyme and microsatellite markers

As expected, microsatellite markers showed a higher degree of polymorphism than allozymes with, for instance, a 20-fold difference in the observed proportion of heterozygous individuals at a given locus. As a consequence, microsatellites will constitute better tools for statistically intensive estimations based on the detection of heterozygotes, such as that of the mating system of sorghum using progeny assays (Ritland and Jain 1981). Likewise, in a detailed comparative analysis of microsatellite and allozyme markers in brown trout, Estoup et al. (1998) have observed that the higher level of polymorphism of the former resulted in a higher power of tests for population differentiation, genotypic linkage disequilibrium, and isolation by distance. Nevertheless, allozymes are still better suited for genetic-diversity assessments because a large body of literature is available for comparison with other gene pools or species (Hamrick and Godt 1997), whereas microsatellite data are inevitably subject to bias due to the fact that loci are chosen based on their polymorphism.

Our results showed that relative measures of genetic structure, such as Wright's inbreeding coefficients F_{IS} and F_{IT}, and Nei's coefficient of genetic differentiation G_{ST}, are of the same order of magnitude for the two sets of markers, though with microsatellites indicating stronger differentiation among fields than allozymes. In the study mentioned above, Estoup et al. (1998) observed that multilocus F_{ST} estimates of differentiation over the entire set of populations investigated were not significantly different for both categories of markers. In contrast, Streiff et al. (1998) reported a stronger genetic structure in oaks when inferred from microsatellite data as compared to allozymes, but pointed out that the main difference between the two markers resides in the interlocus variance in F_{ST}, which is much larger for allozymes. Due to the low number of microsatellite loci used in the present study, interlocus variances can not be validly compared.

Substantial discrepancies between the two types of markers were observed in the estimation of the degree of variation among fields in within-field gene diversity, with allozymes showing seven times more heterogeneity than microsatellites. Similarly, based on data published by Estoup et al. (1998), we computed that the coefficient of variation in estimates of H_e for populations of brown trout was two-times higher for allozymes than for microsatellites. Schoen and Brown (1991) pointed out that conservation strategies should take into account the amount of intraspecific variation in population genetic diversity, which is generally larger in selfing species as compared to outbreeders. It may seem paradoxical that this measure is influenced to such a great extent by the type of marker. We suggest that the discrepancy could be due to the large variance of estimation of gene diversity among allozyme loci, causing allozyme estimates for individual fields to be quite unreliable. Alternatively, this could result from the high mutation rates of microsatellites leading to a homogenisation of variation after historical events such as founder events or population bottlenecks. Further studies are needed to show how to obtain a valid estimate of the heterogeneity in H_e that could be used in conservation management.

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