

Survey of efficiency of six microsatellite loci in Iranian indigenous cattle and buffalo populations

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Abstract

Genetic diversity of three native buffalo populations from Azarie, Mazandaranie and Khuzestanie and two Iranian cattle breeds namely Sistanie and Taleshie were estimated using six microsatellite markers. Thirty individuals were randomly selected from each population/breed. Total genomic DNA was extracted by an optimized phenol-chloroform extraction method. The extracted DNA was amplified through polymerase chain reaction (PCR). Of the six microsatellite loci used in this study, two loci (ETH10 and ETH 225) were monomorphic within the three buffalo populations. Genetic distance between the populations was estimated by F_{ST} (two by two) method. Maximum genetic distance was observed between Khuzestanie and Mazandaranie buffalo populations (55%); whereas the minimum genetic distance (31%) was observed between Khuzestanie and Azarie populations. Values of both polymorphic information content (PIC) and heterozygosity (observed and expected) were higher within two cattle breeds as compared to those estimated for three buffalo populations.

Keywords: Genetic diversity; Polymorphism; Microsatellite markers; Buffalo; Cattle.

INTRODUCTION

Biodiversity among domestic animals in developing countries is enormous. However, with the introduction of superior animal breeds with excellent performance, the native animal resources with good adaptability but lower productivity are in great danger. The list of

extinct local breeds and deteriorated remaining ones are becoming longer every year. Erosion of genetic diversity in a breed may cause increase in the rate of inbreeding and genetic abnormalities, thereby decrease in animal performance, particularly for reproductive traits. These will virtually reduce the global gene pool for future development and can be considered as a serious threat for universal food security. Therefore, the urgency and need for conservation of genetic resources in animal biodiversity is clear, particularly for those in the developing countries. Genetic conservation programs require the thorough evaluation of the existing diversity at both local and international levels (Hall *et al.*, 1995).

During the past two decades the genetic markers, especially DNA markers such as microsatellites or short simple repeats (SSRs) have become the mainstay of fingerprinting and genetic diversity assessments (Goldstein *et al.*, 1998). Microsatellites are ubiquitous, accessible and highly variable components of eukaryotic genomes and as such seem to be ideal tools for population genetic studies of the nuclear genome (Jarne and Lagoda, 1995).

Animal scientists have introduced buffalo as a "future livestock" and claim that their potentials will be more than any other livestock (Van Hooft *et al.*, 2000). Buffalo plays an important role in the rural economy of Iranian farmers particularly in the remote areas. However, in spite of its importance and apparent genetic diversity, molecular markers have not been used to find out the level of diversity in Iranian buffalo population and their link to other populations, such as those in Indian subcontinent. The main goal of this study is to assess the genetic diversity and relationship among three Iranian native buffalo populations and

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two local cattle breeds for better utilization in breeding programs.

MATERIALS AND METHODS

Sample collection and DNA extraction: Blood samples were collected from 30 unrelated animals from each of the three buffalo populations from Azarie, Mazandaranie and Khuzestanie regains and also two cattle breeds: Taleshie and Sistanie. DNA was extracted using an optimized phenol–chloroform extraction method that guarantees long-term stability of DNA samples (Sambrook *et al.*, 1989 and Mommens *et al.*, 1999). Quality assessment of the DNA was done by running on 2% agarose gel along with lambda DNA as marker.

PCR and Electrophoresis: PCR amplification of individual microsatellites was carried out. The total volume of 20 µl PCR mixture contained 50 ng of template DNA, 200 µM of each of dNTPs, 0.3 µM of each primer, PCR buffer (10X), 1 unit *Taq* DNA polymerase and 2 mM MgCl₂. Six microsatellite loci previously used by Loftus *et al.* (1999) namely ETH152, ETH225, INRA005, HEL13, ILSTS005 and ETH10 were used in this study. Microsatellite markers used in this study and their chromosomal positions are listed in Table 1. The reaction mixture was subjected to an initial 4 min denaturation at 94°C, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 54–57°C for 30s, extention at 72°C for 45s and a final

extention step at 72°C for 5 min. Samples were mixed with 5 µl formamide solution and denatured at 93°C. Three microliter of amplification products were loaded onto 6% denaturing polyacrylamide sequencing gels and were stained by silver staining method (Silver sequence TM DNA sequencing system technical manual, 2003). The stained gels were scanned and the bands were scored as 1 for presence and 0 for absence.

Data analysis: POPGENE software was used to estimate the observed and expected heterozygosity (Yeh, *et al.*, 1999). The percentage of observed heterozygosity was calculated for each population. Average expected theoretical heterozygosity from Hardy-Weinberg assumptions was calculated using the formula (Hedrick, 1999):

$$H_e = 1 - \sum_{i=1}^n p_i^2$$

Where:

p_i as the *i*th allele frequency.

Polymorphic information content (PIC) was calculated using the formula (Botstein *et al.*, 1980)

$$PIC = 1 - (\sum_{i=1}^k p_i^2) - \sum_{i=1}^{k-1} \sum_{j=i+1}^k 2p_i^2 p_j^2$$

Where:

p_i, p_j are frequencies of corresponding alleles. Effective number of alleles (n_e) was calculated using the formula (Hedrick, 1999):

$$n_e = 1 / \sum_{i=1}^n p_i^2$$

Table 1. Sequence, repeating motifs and amplicon size of the primers used.

| Locus | Repeated motif | Chr.** | Primer sequences(5'-3') | Size range(bp) |
|----------|--|--------|--|----------------|
| ILSTS005 | (TG) ₄ (TACA)(TA) ₅ -(TG) ₉ (TA)(TG) ₂ (TA) ₇ | 10 | GGA AGC AAT GAA ATC TAT AGC C TGT TCT GTG AGT TTG TAA GC | 181-193 |
| INRA005 | (CA) ₁₃ | 12 | CAA TCT GCA TGA AGT ATA AAT AT CTT CAG GCA TAC CCT ACA CC | 137-143 |
| HEL13 | (GA) ₄ (CA) ₅ TA(CA) ₁₈ | 11 | TAA GGA CTT GAG ATA AGG AG CCA TCT ACC TCC ATC TTA AC | 177-197 |
| ETH10 | (CA) ₁₉ | 5 | GTT CAG GAC TGG CCC TGC TAA CA CCT CCA GCC CAC TTT CTC TTC TC | 210-226 |
| ETH225 | (TG) ₄ (N*) ₄ (CA) ₁₈ | 9 | GAT CAC CTT GCC ACT ATT TCC T ACA TGA CAG CCA GCT GCT ACT | 140-156 |
| ETH152 | (CA) ₁₇ | 5 | TAC TCG TAG GGC AGG CTG CCT G GAG ACC TCA GGG TTG GTG ATC AG | 191-202 |

*N: any of the four nucleotides A,C,G and T

**Chr: No. of chromosome

This parameter gives an indication of the relative influence of the alleles.

NTSYS pc 2.02 was used for estimating of Principal Component Analysis (PCA) and constructing of dendrogram. PIC was estimated, using HET software package (Ott, 1989). Genetic distance between populations was estimated by F_{ST} two by two using Arlequin 2.00 software package (Schneider *et al.*, 1992).

RESULTS

DNA was extracted from 30 blood samples each from three buffalo populations and two cattle breeds. PCR amplification was carried out using six microsatellite specific primers listed in table 1. The size of the amplified bands using different microsatellite specific primers ranged between 137 bp (INRA005) to 226 bp (ETH10). Polymorphism was observed at all of these loci. However, the three buffalo populations were monomorphic at ETH225 and ETH10 loci. Figure 1 shows the PCR products at ILSTS005 locus in Azarie

population. Allelic diversity and heterozygosity is shown in this population as an example.

Hardy-Weinberg Equilibrium: Chi square (χ^2) test was used to evaluate Hardy-Weinberg equilibrium in (HWE) 24 out of 30 alleles at six loci (monomorphic loci were not included in three buffalo populations). Results showed that HEL13 locus in the three buffalo populations, ETH152 in Mazandaranie and Azarie buffalo populations, ETH152, ETH225 and INRA005 in each cattle population were found to be deviating from HWE equilibrium ($p < 0.05$).

Between cattle breeds, the maximum number of alleles (7 alleles) were observed at ETH225 locus in Taleshie population and the minimum number of alleles (3 alleles) were observed at INRA005 locus in Sistanie population (Table 2).

Also, among buffalo populations the maximum number of alleles (7 alleles) were observed at ETH152 locus in Khuzestanie population and the minimum number of alleles (1 allele) observed at monomorphic loci in three buffalo populations (Table 3). The mean number of alleles between cattle breeds (4.6 and 5.0)

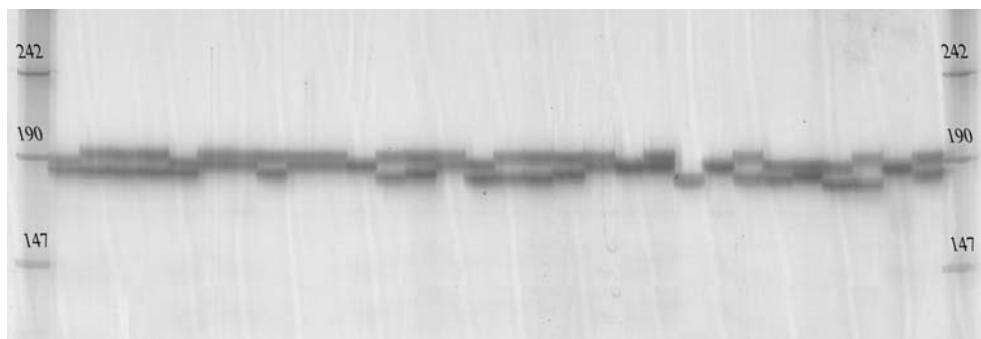


Figure 1. The PCR products obtained using ILSTS005 primer run on 6% denaturing PAGE and stained by silver stain in Azarie buffalo population (Lane 2 to 31). Lanes 1 and 32 are molecular weight markers.

Table 2. Effective and observed number of alleles in microsatellite loci in two cattle.

| Locus | Sistanie | | Taleshie | | Total | |
|-----------------|-----------------------|-----------------------------------|-----------------|----------------------|--------------|----------------------|
| | <i>n</i> ^a | <i>n_e</i> ^b | <i>n</i> | <i>n_e</i> | <i>n</i> | <i>n_e</i> |
| ILSTS005 | 4 | 2.8 | 5 | 3.2 | 7 | 4.2 |
| ETH152 | 6 | 3.3 | 5 | 3.4 | 9 | 2.7 |
| ETH225 | 6 | 3.8 | 7 | 4.2 | 9 | 4.9 |
| INRA005 | 3 | 2.0 | 4 | 2.8 | 4 | 2.5 |
| HEL13 | 5 | 3.2 | 5 | 3.0 | 7 | 3.5 |
| ETH10 | 4 | 2.6 | 4 | 2.3 | 4 | 3.9 |
| Mean | 4.6 | 2.9 | 5 | 3.1 | 6.7 | 3.6 |

^a number of observed alleles.

^b number of effective alleles.

Table 3. Effective and observed number of alleles in microsatellite loci in buffalo populations.

| Locus | Khozestanie | | Mazandaranie | | Azarie | | Total | |
|-----------------|----------------|-----------------------------|--------------|----------------|--------|----------------|-------|----------------|
| | n ^a | n _e ^b | n | n _e | n | n _e | n | n _e |
| ILSTS005 | 3 | 1.9 | 2 | 1.7 | 4 | 2.5 | 4 | 3.1 |
| ETH152 | 7 | 3.4 | 5 | 2.9 | 6 | 3.6 | 7 | 2.9 |
| ETH225 | 1 | 1 | 1 | 1.0 | 1 | 1.0 | 1 | 1.0 |
| INRA005 | 2 | 1.8 | 3 | 2.0 | 3 | 2.3 | 3 | 2.1 |
| HEL13 | 4 | 3 | 4 | 2.1 | 6 | 3.0 | 6 | 3.4 |
| ETH10 | 1 | 1 | 1 | 1.0 | 1 | 1.0 | 1 | 1 |
| Mean | 3 | 2 | 2.6 | 1.8 | 3.5 | 2.2 | 2.4 | 3.7 |

^a: number of observed alleles.^b: number of effective alleles.**Table 4.** The average observed and expected heterozygosity at different microsatellite loci in indigenous cattle and buffalo populations of Iran.

| Locus | Cattle | | | | Buffalo | | | | | |
|-----------------------|----------|-------|----------|-------|-------------|-------|-------------|-------|--------|-------|
| | Sistanie | | Taleshie | | Khozestanie | | Mazandarnie | | Azarie | |
| | Ho | He | Ho | He | Ho | He | Ho | He | Ho | He |
| ILSTS005 | 0.600 | 0.634 | 0.563 | 0.656 | 0.567 | 0.494 | 0.220 | 0.324 | 0.567 | 0.610 |
| ETH152 | 0.633 | 0.710 | 0.602 | 0.714 | 0.633 | 0.733 | 0.670 | 0.757 | 0.700 | 0.723 |
| ETH225 | 0.703 | 0.723 | 0.700 | 0.744 | 0 | 0 | 0 | 0 | 0 | 0 |
| INRA005 | 0.219 | 0.367 | 0.463 | 0.558 | 0.300 | 0.427 | 0.393 | 0.504 | 0.537 | 0.569 |
| HEL13 | 0.521 | 0.567 | 0.543 | 0.690 | 0.600 | 0.695 | 0.467 | 0.514 | 0.633 | 0.678 |
| ETH10 | 0.400 | 0.512 | 0.532 | 0.702 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mean | 0.530 | 0.571 | 0.567 | 0.677 | 0.350 | 0.389 | 0.291 | 0.350 | 0.406 | 0.438 |
| Standard error | 0.068 | 0.054 | 0.049 | 0.065 | 0.027 | 0.032 | 0.029 | 0.028 | 0.032 | 0.034 |

was higher than those estimated in buffalos (3, 3.5 and 2.6). The average effective number of alleles was estimated as 3.1 in Taleshie and 2.9 in Sistanie cattle populations (Table 2). The maximum and minimum mean of effective number of alleles was obtained for Azarie (2.2) and Mazandaranie (1.8) populations respectively (Table 3).

Heterozygosity: Table 4 shows the average observed (H_o) and expected heterozygosity (H_e) and their standard errors for all populations under study.

Maximum value of expected heterozygosity between cattle was 0.744 at ETH1225 locus in Taleshie breed and the minimum expected heterozygosity, 0.367 belonged to INRA005 locus in Sistanie breed.

The highest and the lowest expected heterozygosity belonged to ETH152 locus in Khozestanie population (0.773) and ILSTS005 locus in Mazandaranie population (0.3) respectively (except monomorphic loci). These two loci had the most (7) and the least (2)

observed number of alleles in buffalo populations. In this study, the highest and the lowest expected heterozygosity belonged to the Azarie (0.438) and Mazandaranie (0.350) populations respectively. They have the most and the least intra population genetic diversity respectively.

Polymorphic Information Content (PIC). PIC was estimated using allele frequencies in each polymorphic microsatellite locus (Table 5). The results showed that the mean PIC values in cattle are more than that in buffaloes. The highest and the lowest PIC values belonged to ETH225 locus in Taleshie (0.6846) and INRA005 in Sistanie breeds (0.3010) respectively. In Buffalo populations in our study the highest and the lowest PIC values belonged to ETH152 locus in Khozestanie (0.7102) and ILSTS005 locus in Mazandaranie (0.3015) populations respectively.

Genetic distance and Cluster analysis. The genetic

Table 5. Estimated PIC values in 6 microsatellite loci in Iranian indigenous cattle and buffalo populations.

| Locus | Cattle | | Buffalo | | |
|-----------------|----------|----------|-------------|--------------|--------|
| | Sistanie | Taleshie | Khozestanie | Mazandaranie | Azarie |
| ILSTS005 | 0.5981 | 0.6436 | 0.3680 | 0.3015 | 0.5406 |
| ETH152 | 0.6565 | 0.6519 | 0.7102 | 0.6043 | 0.6739 |
| ETH225 | 0.6838 | 0.6846 | 0 | 0 | 0 |
| INRA005 | 0.3010 | 0.4829 | 0.3318 | 0.4133 | 0.5013 |
| HEL13 | 0.4928 | 0.6282 | 0.6182 | 0.4778 | 0.6016 |
| ETH10 | 0.4866 | 0.5720 | 0 | 0 | 0 |
| Mean | 0.5364 | 0.6105 | 0.3380 | 0.2994 | 0.3682 |

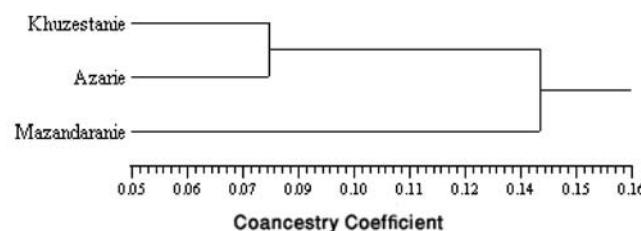
Table 6. Genetic distance between three buffalo populations.

| | Khozestanie | Mazandaranie |
|--------------|-------------|--------------|
| Mazandaranie | 0.55* | 0.00 |
| Azarie | 0.31* | 0.41* |

* P < 0.05

distance among populations was estimated using F_{ST} two by two index (Table 6). This index is considered as an appropriate and common standard index for estimating the differences among populations (Schneider *et al.*, 1992).

Cluster analysis was implemented according to UPGMA by coancestry coefficients. The matrix of coancestry coefficients was estimated using the Arlequin software. It was observed that the Khuzestanie and Azarie populations are more related to each other and Mazandaranie and Khuzestanie had the highest genetic distance among buffalo populations (Fig. 2).

**Figure 2.** The relationship among the buffalo populations from regions of Iran shown by dendrogram.

Multivariate statistical analysis: To condense the genetic variation revealed with the panel of using microsatellite, principal component analysis (PCA) was performed. PCA involves a linear transformation of the observed allele frequencies where the coeffi-

cients are chosen so as to maximize the variation of the transformed data measured along each new coordinate axis (PC). The first and second principal components (PCs), by definition, are the most informative and these were plotted on a two or three dimensional scatter diagram for all using populations. In buffalo populations, the first PC (37.70) and second PC (46.34) was estimated. Figure 3 describes the first and second PC for the microsatellite frequency distributions in the three buffalo populations analyzed.

DISCUSSION

The calculated number of alleles showed higher allelic divergence between the cattle in comparison with those of the buffalo populations. More alleles have been reported for different cattle breeds at these 6 loci (MacHugh, 1996). The effective number of alleles between cattle was found to be higher than buffalos. This might be the result of the fact that the primers were originally designed from information available on cattle DNA sequence.

The obtained results from heterozygosity indicated that the loci with more alleles contain higher rate of heterozygosity in both cattle and buffalo species. Since, the average heterozygosity is related to the level of population divergence, therefore we conclude that Taleshie cattle and Azarie buffalo are more diverse as compared to other individuals in their groups. Genetic diversity of three Mediterranean buffalo populations (from Italy, Egypt and Greece) were studied using 13 microsatellite markers (Moioli *et al.*, 2001). The results showed that by increasing the number of alleles at different loci, the mean genetic diversity increased in populations.

The Hardy-Weinberg equilibrium tests showed several deviant locus in both cattle and as well as buffalo species. However, these deviations for each locus

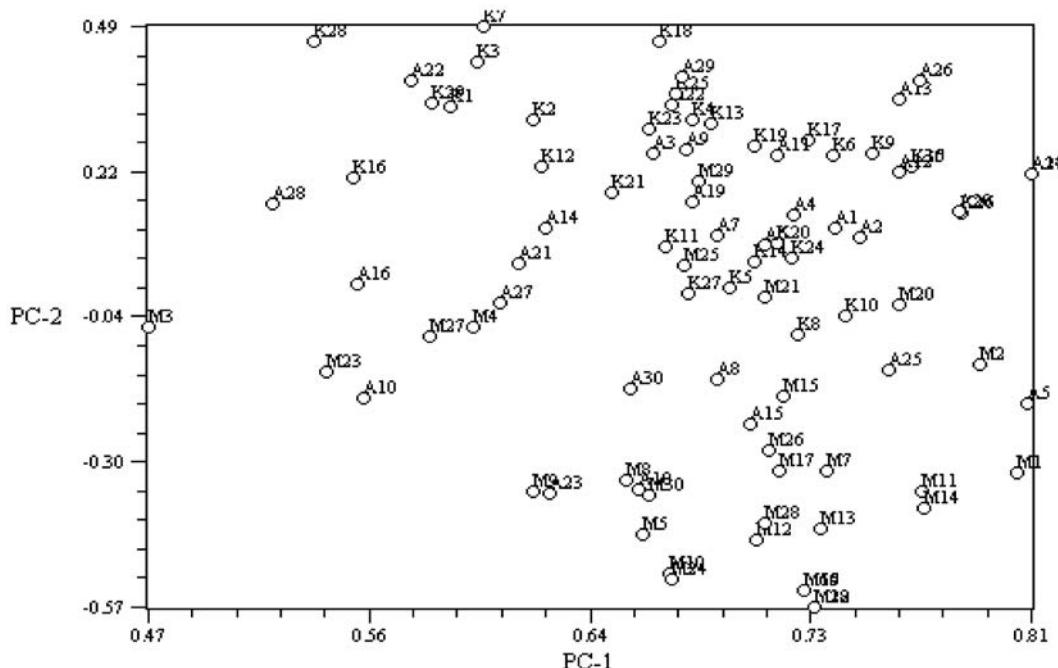


Figure 3. Graph of the first and second principal components PC-1 and PC-2 of the variation exhibited in three buffalo populations studied.

across populations were consistently either positive or negative, indicating locus-specific effects that suggest selection affecting some of these loci. It is possible that such deviations from Hardy-Weinberg equilibrium may result from population substructure and the presence of null alleles (Barker *et al.*, 1997). The use of heterologous primers is likely to increase the incidence of null allele detection. Simonsen *et al.* (1998) in study on the African Buffalo (*Syncerus caffer*) using cattle primers (*Bos taurus*) found that three loci (from six samples) significantly differentiated from Hardy-Weinberg equilibrium, due to an excess of homozygotes, which was explained by them as due to a combination of null alleles and heterologous primers. When primers were redesigned for these loci to make them Buffalo-specific, two out of the three loci were in Hardy-Weinberg equilibrium.

It was found that comparing heterozygosity with PIC, all PIC values were less than their related heterozygosity. It seems that these two parameters are closely related. The polymorphic information content (PIC) is calculated as the expected heterozygosity minus a factor derived from the allele frequencies. Thus PIC must always be less than expected heterozygosity (Botstein *et al.*, 1980). The PIC is a good standard for evaluating genetic markers. PIC has the advantage to incorporate effectively the heterozygote into the formula. A study on the polymorphism at

microsatellite loci in Piedmontese cattle breed showed a relation between the estimated PIC values and the number of alleles as expected. The PIC values in this breed were also lower than their related heterozygosities (Lubieniecka *et al.*, 1999). Similarly, in a study on DNA polymorphism among 4 Belgium cattle breeds using 23 microsatellite markers, it was shown that the derived PIC values were less than the related heterozygosity (Peelman *et al.*, 1998). The relation between PIC and heterozygosity is not direct but they are closely related (Lubieniecka, *et al.*, 1999). Our results were also in agreement with the above studies.

In buffalo populations the PCs separated Mazandaranie population from two other populations. Genetic distance and principal component analysis showed the close genetic relationship between Khuzestanie and Azarie populations. Considering the geographical and climatic differences of the habitats of the two populations this result was expected. Genetic distance between Khuzestanie and Azarie populations was the lowest ($P < 0.05$). Provinces Khuzestan and Azarbaijan that are the origin and habitat of the Khuzestanie and Azarie populations, respectively, are very distinct in terms of climatic conditions such as temperature and humidity. The two provinces are geographically far from each other and the low genetic distance was not expected from this two populations. This apparently unexpected result led us to search for

the origin of the two populations.

According to the information collected from the Azarie buffalo breeders from Orumeyeh region and opinion from Ahwaz agriculture experts, we found that Azarie breeders had selected their young bulls from Khuzestan region. This selection was being conducted since 1963 to improve such as high milk production, milk quality, meat quality and high body strength. The Azarie and Mazanderanie population, have the largest and smallest size of buffalo populations respectively. Small size of population can cause more similarity and reduction of heterozygosity. This could justify the lower genetic diversity observed within Mazanderanie buffalo population. The data from our study revealed that genetic diversity in buffalo was lower than the two cattle breeds. In general, markers with high expected heterozygosity are to be preferred as more informative (MacHugh, 1996).

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