# Allozyme differences between populations of chubbyhead barb (*Barbus anoplus* Weber, 1897) and Marico barb (*B. motebensis* Steindacher, 1894)

**JS Engelbrecht*** **and FH van der Bank**
*Department of Zoology, Rand Afrikaans University, PO Box 524, Auckland Park, 2006, South Africa*

## Abstract

Starch gel-electrophoresis was used to assess genetic differences between two morphologically similar barb species. Two population samples of each species were analysed and polymorphism was detected, in one or both species, at 10 of the 30 protein coding loci examined. Relative mobility differences of alleles among the four populations were found at 20 of these loci (56.7%). We conclude that the extent of genetic differences between the two species supports the present taxonomic status of these species, which were previously thought to be synonymous. The genetic differences between the species and populations are of conservation importance and can be used to study possible migration routes and the evolution of the species.

## Introduction

*Barbus anoplus* was initially described from the Buffels River (Gouritz River System) in the Cape and is the most widely distributed fish species south of the Limpopo River (Jubb, 1967; 1968). This species is mostly limited to altitudes above 900 m in Kwazulu-Natal and the former Transvaal and it is often the only species present in these river sections (Crass, 1964; Gaigher, 1973). Morphologically, this species resembles *B. motebensis* which is endemic to the former Transvaal and occurs in the upper catchments of some Limpopo River tributaries (Fig. 1). The distribution maps of Skelton (1993) and distribution records (former Transvaal Nature Conservation) suggest that the distribution of these two species may overlap in the Steelpoort River catchment (Fig. 1) According to Jubb (1968), *B. motebensis* differs from *B. anoplus* in having a lower caudal peduncle scale count and the breeding males of the former species exhibit numerous conical tubercles on the snout, forehead and the lower jaw. Both Gaigher (1969; 1973; 1976) and Groenewald (1958) experienced difficulties in separating *B. anoplus* from *B. motebensis* and suggested that the two species are synonymous. In the present study the genetic variation within and between four geographically isolated populations was investigated to determine whether *B. anoplus* and *B. motebensis* represent one or more species and to what extent the various populations differ from each other.

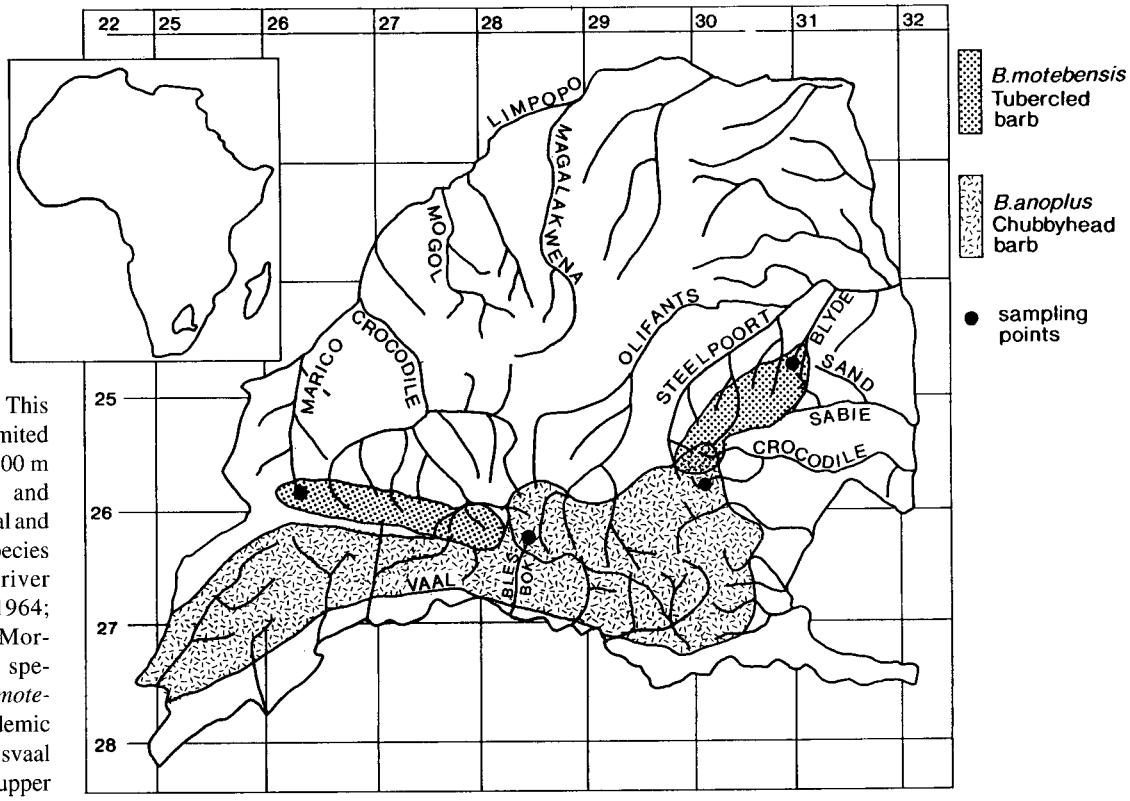


***Figure 1***
*Map of the former Transvaal depicting the distribution of* Barbus motebensis, B. anoplus *and sampling sites*

* To whom all correspondence should be addressed.
Present address: Mpumalanga Parks Board, Private Bag X1088, Lydenburg, 1120, South Africa
☎(01323) 2395; fax (01323) 2732; e-mail:
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**TABLE 1**
**LOCALITIES WHERE *B. ANOPLUS* (A, B) AND *B. MOTEBENSIS* (C, D) POPULATIONS WERE COLLECTED**

| | Species | Locality | Lat. | Long. | River |
|---|---|---|---|---|---|
| 31 | *B. anoplus* | Buffelskloofspruit[a] | 24°47'S | 30°30'E | Crocodile River |
| 25 | *B. anoplus* | Blesbokspruit[b] | 26°11'S | 28°23'E | Vaal River |
| 33 | *B. motebensis* | Ohrigstad River[c] | 24°53'S | 30°36'E | Blyde River |
| 30 | *B. motebensis* | Kaaloog se Loop[d] | 25°47'S | 26°24'E | Marico River |

[a] Tributary of the Crocodile River (Incomati River System)
[b] Tributary of the Vaal River (Orange River System)
[c] Tributary of the Olifants River (Limpopo River System)
[d] Tributary of the Marico River (Limpopo River System)

## Materials and methods

Fifty-six *B. anoplus* specimens were collected from the Crocodile and Vaal Rivers and 63 *B. motebensis* specimens were sampled from the Marico and Ohrigstad Rivers (Table 1). Figure 1 shows the distribution of the two species in the former Transvaal and the sampling points used in this study. Samples were analysed by starch gel-electrophoresis as described by Engelbrecht and Van der Bank (1994).

Average heterozygosity (**H**) was calculated according to Nei (1978) and exact probabilities were used to determine possible deviations of allele frequencies from expected Hardy-Weinberg proportions (Elston and Forthofer, 1977; Swofford and Selander, 1981). Different fixation indices were used to analyse genetic differentiation between populations (Wright, 1978) using **BIOSYS-1** (Swofford and Selander, 1981): where $\mathbf{F_{IT}}$ and $\mathbf{F_{IS}}$ are the fixation indices of individuals relative to the total population and its subpopulation, respectively and $\mathbf{F_{ST}}$ measures the amount of differentiation among subpopulations relative to the limiting amount under complete fixation. We also calculated genetic variance for each level of hierarchy with the **WRIGHT78** procedure (Swofford and Selander, 1981), using the formula of Wright (1978). According to Swofford and Selander (1981), this method is similar to gene diversity analysis used by Nei (1973). The genetic distances of Nei (1972; 1978), **D** (standard) and **D'** (unbiased) respectively and the Cavalli-Sforza and Edwards' (1967) chord distances (**Dc**) were calculated between populations.

Phylogenetic relationships were determined using a phenetic approach and **Dc** values, the **DISWAG** routine (Swofford and Selander, 1981) and a cladistic approach. A cladogram was constructed by phylogenetic analysis using parsimony (**PAUP**)(Swofford, 1985). The latter procedure was used with an allelic frequency data matrix transformed into a presence/absence matrix (allele present in sample = 1 and absent = 0) and the **CLOSEST**, **MULPARS** and **LUNDBERG** algorithms. This program is guaranteed to find the shortest (most parsimonious) tree (Swofford, 1985) and it was preferred to **FREQPARS** (Swofford and Berlocher, 1987), because analysis using the latter method produces a completely bifurcating tree that is confusing when analysing only four populations to compare the two species.

## Results

The 21 enzymes studied produced interpretable results at 29 protein coding loci. The enzyme commission numbers, names of the proteins giving interpretable results, locus abbreviations and buffers giving the best results are presented in Table 2. Polymorphism was detected at 10 loci (34%) in the four populations studied and mobility differences of alleles were present at 20 (69%) of the protein coding loci.

The relative allele mobilities at loci where differences between populations occurred, allelic frequencies and exact significance probabilities for polymorphic loci, as well as average heterozygosity (**H**) values and standard errors are presented in Table 3. All four populations displayed identical allele mobilities at **ADH, AK, GAPDH, IDDH, IDHP, LDH-2** and **PEPA**. Allele mobility differences separating the two species or four populations from one another were detected at the **AAT-1, -2, CK, EST-1, -2, -3, PGDH, GPI-1, -2, MDH-1, -2, MPI, PGDH, PROT-1, -2, -3, -5** and **SOD** protein coding loci.

Relatively low exact significance probabilities for alleles that deviated from expected Hardy-Weinberg proportions were encountered at 30% of the polymorphic loci studied (Table 3). Deviations from expected Hardy-Weinberg proportions were evident at the **ME** protein coding locus for all four populations studied; **AAT-1, GPI-2** and **PROT-2** for the population from the Vaal River; **EST-1** for the Crocodile River population; **PEPS** for the Crocodile and Ohrigstad River populations (Table 3). Average heterozygosity values for the *Barbus* populations studied, based on 30 protein coding loci, ranged between 0.038 and 0.076 (Table 3). **F**-statistics mean values of -0.008, 0.849 and 0.850 were calculated for **FIS, FIT** and **FST** respectively. The genetic variance values were 6.15, 8.25 and 2.10 for *locality-species, locality-total* and *species-total* analysis respectively.

Genetic distance (**D**) between the two *B. anoplus* and the two *B. motebensis* populations averaged 0.687. Smaller **D** values (0.230 and 0.329) were found between the populations within the *B. anoplus* and *B. motebensis* populations respectively (Table 4). Values obtained by using various other coefficients displayed a similar trend (Table 4). The phenetic tree (Fig. 2a) obtained by using **DISWAG**, rooted at the midpoint of greatest patristic distance and based on **Dc** values (Table 4), illustrates the genetic differences between the barb populations studied and clearly shows the existence of two separate groups, namely a chubbyhead (*B. anoplus*) and a tubercled barb group (*B. motebensis*). A single cladogram was obtained using **PAUP** (Fig. 2b), which is almost identical to the grouping produced by the phenogram (Fig. 2a). This is probably a result of the high genetic divergence between populations and the relatively small influence of polymorphic gene frequencies on genetic distances between these populations.

## Discussion

Deviations from expected Hardy-Weinberg proportions were encountered at 30% of the polymorphic loci studied (Table 3). Perfect Hardy-Weinberg populations do not actually exist in

**TABLE 2**
**ENZYME COMMISSION NUMBERS, PROTEINS EXAMINED, ABBREVIATIONS USED FOR LOCI RESOLVED AND BUFFERS GIVING BEST RESULTS. LOCI NOMENCLATURE ACCORDING TO SCHAKLEE *ET AL.* (1990)**

| E.C. No | Enzyme | Locus | Buffer |
|---|---|---|---|
| 2.6.1.1 | Aspartate aminotransferase | **AAT-1, -2** | MF |
| 1.1.1.1 | Alcohol dehydrogenase | **ADH** | RW |
| 2.7.4.3 | Adenylate kinase | **AK** | TC |
| 2.7.3.2 | Creatine kinase | **CK** | RW |
| 3.1.1.1 | Esterase | **EST-1, -2 , -3** | MF |
| - - - - | General (unidentified) protein | **PROT-1, -2, -3, -4, -5** | MF |
| 5.3.1.9 | Glucose-6-phosphate isomerase | **GPI-1, -2** | MF |
| 1.2.1.12 | Glyceraldehyde-3-phosphate dehydrogenase | **GAPDH** | RW |
| 1.1.1.8 | Glycerol-3-phosphate dehydrogenase | **G3PDH** | MF |
| 1.1.1.14 | L-Iditol dehydrogenase | **IDDH** | RW |
| 1.1.1.42 | Isocitrate dehydrogenase ($NADP^+$) | **IDHP** | TC |
| 1.1.1.27 | L-Lactate dehydrogenase | **LDH-1, -2** | TC, MF |
| 1.1.1.37 | Malate dehydrogenase | **MDH-1, -2** | RW |
| 1.1.1.40 | Malic enzyme ($NADP^+$) | **ME** | MF |
| 5.3.1.8 | Mannose-6-phosphate isomerase | **MPI** | MF |
| 3.4.-.- | Peptidase | | |
| | Dipeptidase | **PEPA** | MF |
| | Peptidase-S | **PEPS** | |
| 5.4.2.2 | Phoshoglucomutase | **PGM** | RW |
| 1.1.1.44 | Phosphogluconate dehydrogenase | **PGDH** | MF |
| 1.15.1.1 | Superoxide dismutase | **SOD** | RW |

**MF**: continuous Tris, boric acid, EDTA buffer (pH 8.6) described by Markert and Faulhaber (1965).
**RW**: discontinuous Tris, citric acid (gel pH 8.7), lithium hydroxide, boric acid (tray pH 8.0) buffer system (Ridgway et al., 1970).
**TC**: continuous Tris, citric acid (pH 6.9) buffer system (Whitt, 1970).

nature and departures from Hardy-Weinberg proportions may occur because of several factors such as the Wahlund (1928) effect, natural selection, interbreeding and population bottlenecks (Ferreira et al., 1984). In the present study the deviations from expected Hardy-Weinberg proportions were mainly caused by a deficiency of heterozygotes. A deficiency of heterozygotes can be the consequence of selection against a heterozygote or a homozygote, which is a common phenomenon within fish populations (Kirpichnikov, 1981). *Barbus anoplus* and *B. motebensis* are mostly confined to the upper catchments of rivers where natural and artificial barriers often subdivide the species into numerous isolated populations. It is therefore possible that these deviations from expected Hardy-Weinberg proportions may be the result of interbreeding in small and isolated populations, causing a reduction of heterozygotes (Chakraborty and Nei, 1977). The **H** values obtained in the present study (0.038-0.079) are slightly lower than those found by Mulder (1989) for large *Barbus* species (0.052-0.216). However, these values are similar to the average **H** value (0.051) given by Nevo et al. (1984) for 183 species of fish and by Engelbrecht and Van der Bank (1994) for small *Barbus* species. According to Berrebi et al. (1990) and Agnèse et al. (1990) small *Barbus* species are diploid while the large *Barbus* species tend to be tetraploid and it is therefore reasonable to assume that the relatively lower **H** values found in small *Barbus* species could be associated with smaller numbers of active loci in diploids. Naran (pers. comm., 1996) found that although most *Barbus* species in Southern Africa are tetraploid or hexaploid, the concerned species are diploid.

The fixation index, $F_{ST}$, quantifies inbreeding due to population subdivision or the reduction of heterozygosity of a subdivision due to genetic drift (Lawson et al., 1989). The $F_{ST}$ value (0.850) over all populations suggests a great genetic differentiation between the populations and is also comparable with $F_{ST}$ values (0.609) found for isolated cave populations of fish of the genus *Astyanyx* (Avise and Selander, 1972).

The genetic variance is similar to the gene diversity analysis of Nei (1973) so that the variance of populations in terms of the total (8.25) will give an indication of total genetic divergence. Most of this divergence is derived from the variance of the populations compared with the species (6.15), which is considerably larger than the variance for species compared with the total variance (2.10). This is indicative of the relatively large genetic differentiation among the four populations and a relatively small genetic differentiation within the populations.

**D** values ranging between 0.230 and 0.798 where observed in the present study among the four populations (Table 4), which compares well with **D** values reported by Mulder (1989) between nine large *Barbus* species. Similar **D** values were also found by Agnèse et al. (1990) between two species of small barbs (0.128) and seven species of large barbs (0.086 to 0.274), and Berrebi et al. (1990) reported values of between 0.112 and 0.565 for five small barbs. The former authors obtained **D** values between two conspecific populations of large *Barbus* species of approximately 0.01. For fish, Schaklee et al. (1982) found that **D** between pairs of conspecific populations ranged from 0.002 to 0.07 (average 0.05) and for congeneric species it ranged from 0.03 to 0.61 (average 0.3). According to Grant and Stahl (1988) the boundaries between taxonomic categories are not sharp but, in general, the

**TABLE 3**
**RELATIVE MOBILITIES (RM), ALLELE FREQUENCIES, AVERAGE HETEROZYGOSITY (*H*), EXACT SIGNIFICANCE PROBABILITIES VALUES (*P*) FOR POLYMORPHIC LOCI AND LOCI WHERE MOBILITY DIFFERENCES WERE DETECTED BETWEEN *B. MOTEBENSIS* AND *B. ANOPLUS* POPULATIONS**

| | | *Barbus motebensis* | | *Barbus anoplus* | |
|---|---|---|---|---|---|
| **Locus** | **RM** | **Ohrigstad** | **Marico** | **Crocodile** | **Vaal** |
| AAT-1 | 100 | 1.000 | 1.000 | - | - |
| 90 | - | - | 1.000 | 0.652 | |
| 80 | - | - | - | 0.348 | |
| *P* | | | | *0.004* | |
| AAT-2 | 100 | 1.000 | - | - | - |
| 90 | - | - | 1.000 | 1.000 | |
| 00 | - | 1.000 | - | - | |
| CK100 | 1.000 | 1.000 | - | - | |
| 90 | - | - | 1.000 | 1.000 | |
| EST-1 | 100 | - | - | 0.952 | 1.000 |
| 90 | 1.000 | 1.000 | - | - | |
| 80 | - | - | 0.048 | - | |
| *P* | | | *0.049* | | |
| EST-2 | 100 | 1.000 | - | 1.000 | 1.000 |
| 90 | - | 1.000 | - | - | |
| EST-3 | 100 | - | - | 1.000 | 1.000 |
| 90 | - | 1.000 | - | - | |
| 00 | 1.000 | - | - | - | |
| G3PDH | 100 | 0.970 | 1.000 | 1.000 | 0.900 |
| 90 | 0.030 | - | - | 0.100 | |
| *P* | *1.000* | | | *1.000* | |
| GPI-1 | 100 | - | - | - | 1.000 |
| 90 | 0.030 | 1.000 | - | - | |
| 80 | .970 | - | - | - | |
| 70 | - | - | 1.000 | - | |
| *P* | *0.015* | | | | |
| GPI-2 | 100 | - | - | - | 0.300 |
| 90 | - | 1.000 | - | 0.700 | |
| 80 | 1.000 | - | - | - | |
| 70 | - | - | 0.984 | - | |
| 60 | - | - | 0.016 | - | |
| *P* | | | *1.000* | *0.012* | |
| LDH-1 | 100 | 0.985 | 1.000 | 1.000 | 1.000 |
| 90 | 0.015 | - | - | - | |
| *P* | *1.000* | | | | |
| MDH-1 | 100 | 1.000 | - | - | - |
| 90 | - | 1.000 | 1.000 | 1.000 | |
| MDH-2 | 110 | 1.000 | - | - | - |
| 100 | - | 1.000 | 1.000 | 1.000 | |
| ME | 100 | 0.621 | 0.417 | 0.515 | 0.700 |
| 90 | 0.379 | 0.583 | 0.485 | 0.300 | |
| *P* | *0.284* | *0.256* | *0.308* | *0.060* | |
| MPI | 100 | 1.000 | 1.000 | - | 1.000 |
| 90 | - | - | 1.000 | - | |
| PEPS | 100 | 0.561 | 0.650 | 0.581 | 0.900 |
| 90 | 0.439 | 0.350 | 0.419 | 0.100 | |
| *P* | *0.078* | *1.000* | *0.139* | *1.000* | |
| PGDH | 100 | - | - | 1.000 | - |
| 90 | 1.000 | 1.000 | - | 1.000 | |
| PGM | 100 | - | - | - | 0.100 |
| 90 | 1.000 | 1.000 | 1.000 | 0.900 | |
| *P* | | | | *1.000* | |

distances between conspecific populations are larger than 0.05 and average about 0.40 for congeneric species. The average value of **D** in the present study between *B. motebensis* and *B. anoplus* (0.687) falls within the upper range for congeneric species as discussed above, supporting the present taxonomic status of separate species. It is likely that sympatric *B. anoplus* and *B. motebensis* communities can occur and that it could have caused some confusion concerning the specific status of the two species. The genetic trees (Fig. 2a and b) also depict two groups of genetically different barbs, namely a chubbyhead group (*B. anoplus*) and a tubercled barb group (*B. motebensis*). The comparatively high **D** value (0.329) found between the two *B. motebensis* populations also falls within the range for congeneric species, mainly as a result of relative mobility differences of monomorphic (fixed) alleles at the **AAT-2, EST-2, -3 GPI-1, -2, MDH-1, -2** and **PROT-5** protein coding loci (Table 3). The unexpectedly high level of divergence between the two *B. anoplus* populations examined (**D**=0.230) on the other hand is the result of mobility differences at fixed alleles at the **GPI-1, -2, MPI** and **PGDH** protein coding loci (Table 3). The presence of these biochemical markers should be investigated in relation to other *B. anoplus* populations within its wide distribution range as well as other taxonomically associated barb species from Southern Africa (e.g. *B. amatolicus* and *B. gurney)* to determine the taxonomic significance of these differences. Morphological differences between geographically subdivided *B. anoplus* populations was detected by Barnard (1943), who divided the chubbyhead barbs into two species namely *B. karkensis*, from Natal and *B. anoplus* for the rest of its distribution. Barnard (1943) also subdivided *B. anoplus* into three geographically isolated forms (Orange, Olifants and Gouritz River Systems) substantiated by morphological differences among the three forms. The genetic differences between the *B. anoplus* populations found in the present study suggest that the morphological subdivision by Barnard (1943) may be substantiated by a more detailed study of the genetic differences between these geographically subdivided *B. anoplus* populations.

These results also suggest that the dispersion and isolation of these fish species into the different rivers of Southern Africa have created ideal

conditions for genetic divergence and speciation. These conditions would result in allopatric speciation, which is a very common phenomenon in fish populations (Bush, 1975). A subdivided population structure may result in a faster rate of adaptive morphological evolution and a founder effect in such populations will most likely lead to genetic changes (Templeton, 1980). According to the latter author, an adaptive divergence mode of speciation can be present where populations are divided by intrinsic barriers, as with the present study. Since the mutation process is random and selection always interacts to some degree with genetic drift, ordinary micro-evolutionary processes lead to adaptive divergence between isolated populations even if they inhabit almost identical environments. However, the rate of adaptive divergence can be greatly increased if the environments are also different. *B. anoplus* and *B. motebensis* populations are often isolated in the upper catchments of rivers where movement or contact between these populations is limited to geological stream capturing events. The relatively large genetic variation observed between the populations studied is most likely the result of such isolation and suggests that the concerned species may consist of a myriad of conspecific and subdivided populations that are genetically distinct and of significance for the maintenance of biodiversity and the continued evolution of the species. A more detailed study of the genetic variation between selected populations within these species could therefore be used to study microevolution, speciation and migration of fish in South Africa on a timescale that coincides with known geological events. The results of the present study emphasise the need for increased efforts towards a concerted campaign to characterise our flora and fauna genetically.

**TABLE 3 (CONTINUED)**

| Locus | RM | *Barbus motebensis* Ohrigstad | *Barbus motebensis* Marico | *Barbus anoplus* Crocodile | *Barbus anoplus* Vaal |
|---|---|---|---|---|---|
| PROT-1 | 100 | 1.000 | 1.000 | - | 1.000 |
| 90 | - | - | 1.000 | - | |
| PROT-2 | 100 | 0.136 | - | - | - |
| 90 | 0.864 | 1.000 | - | - | |
| 80 | - | - | - | 0.440 | |
| 70 | - | - | 1.000 | 0.560 | |
| *P* | *1.000* | | | *0.420* | |
| PROT-3 | 100 | 1.000 | 1.000 | - | - |
| 90 | - | - | 1.000 | 1.000 | |
| PROT-4 | 100 | 1.000 | 0.917 | 1.000 | 1.000 |
| 90 | - | 0.083 | - | - | |
| *P* | | *1.000* | | | |
| PROT-5 | 100 | - | 1.000 | 1.000 | 1.000 |
| 00 | 1.000 | - | - | - | |
| SOD | 100 | - | - | 1.000 | 1.000 |
| 10 | 1.000 | 1.000 | - | - | |
| *H* | 0.044 | 0.038 | 0.044 | 0.079 | |
| ó | ± 0.024 | ± 0.023 | ±0.023 | ± 0.029 | |

**TABLE 4**
**GENETIC DISTANCES BETWEEN *B. MOTEBENSIS* AND *B. ANOPLUS* POPULATIONS CALCULATED USING D (NEI, 1972), D'(NEI, 1978) AND DC (CAVALLI-SFORZA AND EDWARDS, 1967)**

| | *Barbus motebensis* Ohrigstad | *Barbus motebensis* Marico | *Barbus anoplus* Crocodile | *Barbus anoplus* Vaal |
|---|---|---|---|---|
| **Marico River** | | | | |
| D | 0.329 | - | | |
| D' | 0.328 | - | | |
| $D_C$ | 0.470 | - | | |
| **Crocodile River** | | | | |
| **D** | 0.798 | 0.660 | - | |
| D' | 0.797 | 0.659 | - | |
| $D_C$ | 0.658 | 0.616 | - | |
| **Vaal River** | | | | |
| **D** | 0.575 | 0.424 | 0.230 | - |
| **D'** | 0.574 | 0.423 | 0.228 | - |
| $D_C$ | 0.596 | 0.530 | 0.416 | - |

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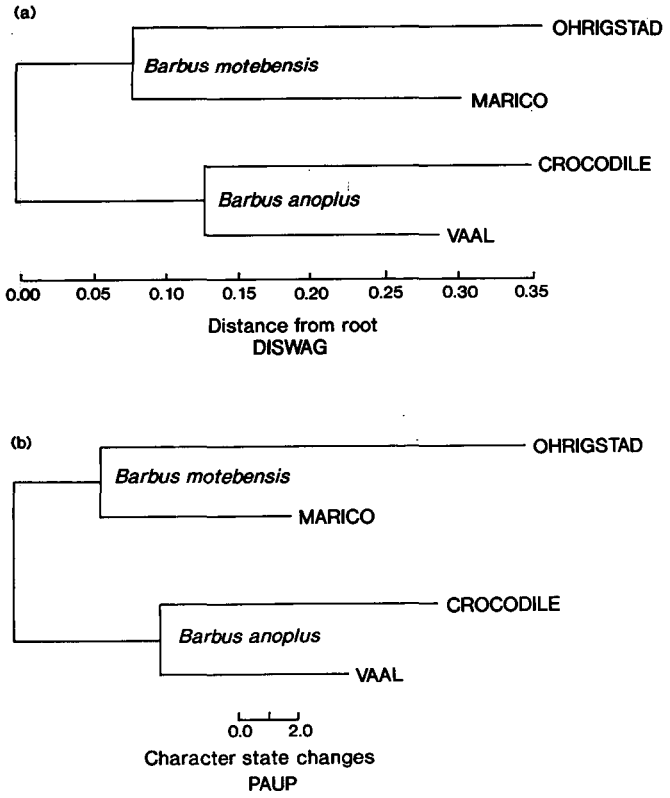


***Figure 2***
*Phylogenetic trees obtained by using a)* ***DISWAG*** *and b)* ***PAUP****, showing the relationship between as well as within populations of* Barbus motebensis *and* B. anoplus

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