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Genetic Diversity and Population Structure of *Kaloula borealis* (Anura, Microhylidae) in Korea

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To assess the genetic diversity and population structure of Korean *K. borealis*, allozyme analysis was performed. The average genetic variability of Korean *K. borealis* populations was %P=13.2, Ho=0.048, and He=0.045. This value was the lowest in comparison with other Korean amphibian species studied. Also, the value was much lower than that of a reference population from Chinese *K. borealis* (%P=50, Ho=0.125, He=0.172). Wright's F-statistics showed that Korean *K. borealis* has distinctly low level of gene flow among regional populations (F_{ST}=0.339, *Nm*=0.487) in comparison with other Korean amphibian species studied. However, the average level of genetic divergence among Korean *K. borealis* populations was moderate (Nei's D=0.020). Therefore, it appeared that low levels of genetic diversity (He=0.045) and gene flow (*Nm*=0.487) among regional populations are probably due to the results of decreasing population size and patchy distribution of this species in Korea.

Kaloula borealis (Barbour, 1908) is distributed in Korea and northeastern, northern, and central China (Zhao and Adler, 1993). In South Korea, the population size of this species is distinctly decreasing and the distribution of this species is patched because of the destruction and pollution of natural habitats. Therefore, this species was designated as a species of specified wildlife by Korean Ministry of Environment.

Genes are the basic material of evolutionary changes and, at the same time, hold in memory the records of events that have occured in the past. Thus, conservation of species is also conservation of the result of an evolutionary process manifested in the genetic and genomic structures of populations. Studies of evolution and genetic variation are clearly essential topics for the development of theory and practice in conservation biology (Loeschcke et al., 1994). However, the detailed studies of genetic population structure for amphbia are rare (Larson et al., 1984; Szymura and Barton, 1991; Goldman and Barton, 1992; Highton and Hedges, 1995; Driscoll, 1998; Yang et al., 1999).

In this study, we performed the genetic analyses using starch gel electrophoresis to assess the genetic diversity and population structure of *K. borealis* in Korea.

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Materials and Methods

Sample collection and protein electrophoresis

A sum of 178 specimens of *K. borealis* was collected from Korea (11 localities) and China (1 locality) (Table 1, Fig. 1). Live specimens were transported to the laboratory and were stored at -70℃ until analyzed. Tissue samples were obtained from liver, heart and skeletal muscle from each specimen and homogenized by a glass homogenizer in the same volume of distilled water and centrifuged at 18,000 rpm for 30 min at 4℃ to obtain the supernatant for electrophoresis. Voucher specimens were fixed in 10% formalin, preserved in 70% ethanol, and deposited at Yang's Collection in Inha University. The supernatant was subjected to horizontal starch gel (12%) electrophoresis and histochemical staining procedures (Selander et al, 1971; Yang et al., 1997: Appedix I).

Data analysis

Multiple loci were numbered sequentially, and alleles were designated alphabetically with "a" being the fastest migrant. Allele frequencies, mean number of alleles per locus (A), percentages of polymorphic loci at both of the 95% and 99% criteria (%P_{95} and %P_{99}), heterozygosity observed from electromorph (Ho), heterozygosity expected from allele frequencies (He), Wright's (1965) F-statistics, Rogers' (1972) genetic similarity (S) and Nei's (1972) genetic distance (D) were calculated using BIOSYS-1 (Swofford and Selander, 1989). Rogers' similarity coefficients were then clustered by the

Table 1. Collection localities, dates, and samples for the analyses of *Kaloula borealis* in Korea and China

Collection localities	Dates	Sample sizes
1. Inchon: Yonghyun-dong, Nam-gu, Inchon-shi	July 4, 1994	23
2. Pochon: Namyu-ri, Igum-myon, Pochon-gun, Kyongki-do	July 1, 1997	13
3. Chochiwon: Seochang-ri, Chochiwon-up, Yonki-gun, Chungchongnam-do	June 27, 1997	11
4. Kwangju: Chunghyo-dong, Buk-gu, Kwangju-shi	June 26, 1997	2
	June 25, 1998	8
5. Wanju: Ungok-ri, Hwasan-myon, Wanju-gun, Chollabuk-do	June 26, 1997	14
6. Yongam: Deokjin-ri, Deokjin-myon, Yongam-myon, Chollanam-do	June 26, 1998	21
7. Sangju: Cheong-ri, Cheongri-myon, Sangju-shi, Kyongsangbuk-do	June 27, 1997	6
8. Kyongju: Yangdong-ri, Kangdong-myon, Kyongju-shi, Kyongsangbuk-do	June 25, 1998	4
	June 23, 1999	2
9. Andong: Sang-ri, Pungsan-up, Andong-shi, Kyongsangbuk-do	July 1, 1997	30
10. Bukcheju: Kosan-ri, Hankyong-myon, Bukcheju-gun, Cheju-do	May 27, 1995	5
11. Namcheju: Daejong-up, Namcheju-gun, Cheju-do	June 20, 1996	29
12. China: Jinan-shi, Shandong-Province, China	July 29, 1995	10

unweighted pair group method using arithmetic average linkage (UPGMA: Sneath and Sokal, 1973) to provide a general estimate of the overall genetic relationships among populations.

F-statistics were performed to ascertain the degree of allele-frequency heterogeneities among regional populations (F_{ST}), and the level of gene flow among the regional populations (*Nm*) was calculated. Chi-square contingency tests were used to determine if F_{IS} and F_{ST} values differed significantly from zero (Waples, 1987). Throughout this paper F_{ST} was used as an estimate of gene flow, based on the relationship between F_{ST} and *Nm*, the product of effective population size and average number of immigrants (Wright, 1951): $Nm=[(1/F_{ST})-1]/4$.

Results

Genetic variability and genetic differentiation

Of the 28 presumptive loci scored (Table 2), 17 loci were monomorphic across all populations of Korean *K. borealis* and the remaining 11 loci (39%) were polymorphic at the $\%P_{99}$ criterion level. In the Chinese *K. borealis* population, 14 loci (50%) were polymorphic.

Based on allelic frequencies listed in Table 2, the degrees of genetic variability on each population of *K. borealis* were calculated (Table 3). The average genetic variability of Korean *K. borealis* populations was $\%P_{95}$= 13.2 (0.0-32.1), Ho=0.048 (0.000-0.094) and He=0.045 (0.000-0.101) and that of Chinese *K. borealis* population is relatively higher ($\%P_{95}$=50.0, Ho=0.125, He= 0.172).

Average genetic similarities (Rogers' S) and distances (Nei's D) among populations of *K. borealis* were estimated (Table 4). The average level of genetic divergence among Korean *K. borealis* populations and between Korean and Chinese *K. borealis* populations was D=0.024 (0.002-0.065), S=0.948 (0.909-0.989) and D=0.036 (0.019-0.053), S=0.896 (0.872-0.914), respec-

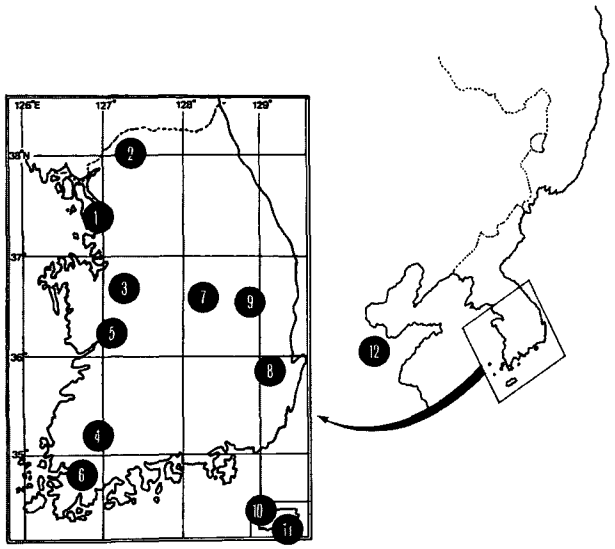

Fig. 1. A map showing the collection localities of 12 populations of *Kaloula borealis* in Korea and China. Numbers refer to collection localities in Table 1.

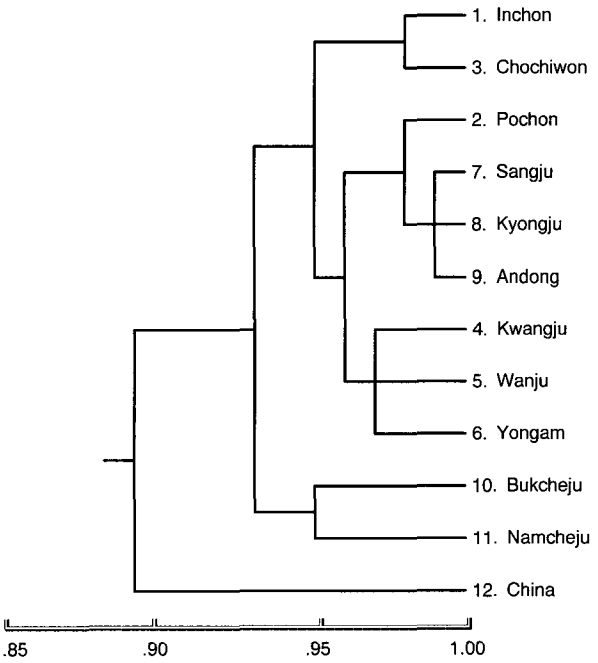

Fig. 2. A dendrogram of 12 populations of *Kaloula borealis* based on Rogers' genetic similarity coefficients.

Table 2. Allele frequencies of 12 populations of *Kaloula borealis* in Korea and China

Locus	Korea											China
	1*	2	3	4	5	6	7	8	9	10	11	12
Xdh	a**	a	a	a	a	a	a	a	a	a	a	a
Ldh-1	a	a	a	a	a	a	a	a	a	a	a	a
Ldh-2	a	a	a	a	a	a	a	a	a	a	a	a
Ipo	a	a	a	a	a	a	a	a	a	a	a	a
Gp-1	a	a	a	a	a	a	a	a	a	a	a	a
Gp-2	a	a	a	a	a	a	a	a	a	a	a	a
Gp-3	a	a	a	a	a	a	a	a	a	a	a	a
Got-1	a	a	a	a	a	a	a	a	a	a	a	a
Lap	a	a	a	a	a	a	a	a	a	a	a	a
Ck	a	a	a	a	a	a	a	a	a	a	a	a
Idh-2	a	a	a	a	a	a	a	a	a	a	a	a
Me-1	a	a	a	a	a	a	a	a	a	a	a	a (0.90)*** b (0.10)
Me-2	a	a	a	a	a	a	a	a	a	a	a	a (0.80) b (0.20)
Got-2	a	a	a	a	a	a	a	a	a	a	a	a (0.95) b (0.05)
Est-1	a	a	a	a	a	a	a	a	a	a	a	a (0.75) b (0.25)
Pgi	b	b	b	b	b	b	b	b	b	b	b	a (0.20) b (0.80)
Gdh	b	b	b	b	b	b	b	b	b	b	b	a (0.10) b (0.90)
αGpd	a	a	a	a	a	a	a	a	a	a (0.95) b (0.05)	a	a (0.80) c (0.20)
Idh-1	b	b	b	b	b	b	b	b	a (0.02) b (0.98)	b	b	b
Pgm	b	b	b	b	b	b	b	b	b (0.98) c (0.17)	a (0.22) b (0.74) c (0.34)	b	a (0.05) b (0.85) c (0.10)
Mdh-1	a	a	a	a	a	a	a	a	a	a (0.95) b (0.05)	a	a
Mdh-2	a	a	a	a	a	a	a	a	a	a (0.98) b (0.02)	a	a
Sdh	b	b	b	b (0.90) d (0.10)	a (0.04) b (0.79) d (0.18)	b (0.81) d (0.19)	b	b	b	b (0.72) c (0.28)	b (0.90) c (0.10)	b (0.80) c (0.10) d (0.10)
Pept	a	a	a	a	a	a	a	a	a	a (0.74) b (0.26)	a (0.90) b (0.10)	a
Est-2	a (0.98) b (0.02)	a	a	a	a	a	a	a	a	a	a	a (0.90) b (0.10)
Est-3	b (0.96) c (0.04)	b (0.81) c (0.19)	a (0.05) b (0.77) c (0.18)	a (0.20) b (0.80)	b (0.93) c (0.07)	a (0.05) b (0.95)	b (0.75) c (0.25)	b	b (0.93) c (0.07)	a (0.03) b (0.94) c (0.03)	b	a (0.10) b (0.50) c (0.40)
Mpi	a (0.67) b (0.33)	b	a (0.68) b (0.32)	b	b	b	a (0.08) b (0.92)	b	a (0.20) b (0.80)	a (0.38) b (0.62)	a (0.60) b (0.40)	a (0.45) b (0.45) c (0.10)
6Pgd	a (0.65) b (0.35)	b	a (0.36) b (0.64)	a (0.15) b (0.85)	a (0.21) b (0.79)	a (0.62) b (0.38)	b	b	b	a (0.76) b (0.24)	a	a (0.35) b (0.65)

*population number, **allele, ***allele frequency

tively (Table 4). The UPGMA clustering based on Rogers' genetic similarities showed that the low level of genetic divergence among Korean *K. borealis* populations as well as between Korean and Chinese *K. borealis* populations (Fig. 2). The clustering also showed considerable corelationship with the pattern of geographic distribution (Figs. 1 and 2).

Genetic population structure and gene flow

In the Korean *K. borealis* populations, the weighted

Table 3. Number of specimens (N), mean number of alleles per locus (A), percentage of polymorphic loci (%P), observed mean heterozygosity (Ho), and Hardy-Weinberg expected mean heterozygosity (He) for 12 populations of *Kaloula borealis* in Korea and China

Population	N	A	%P	Ho	He
1. Inchon	23	1.2	14.3	.043	.052
2. Pochon	13	1.1	7.1	.030	.025
3. Chochiwon	11	1.2	14.3	.068	.065
4. Kwangju	10	1.1	14.3	.046	.046
5. Wanju	14	1.2	14.3	.033	.041
6. Yongam	21	1.1	10.7	.046	.047
7. Sangju	6	1.1	7.1	.012	.021
8. Kyongju	6	1.0	0	.000	.000
9. Andong	30	1.1	7.1	.017	.019
10. Bukcheju	5	1.1	10.7	.029	.033
11. Namcheju.	29	1.4	32.1	.094	.101
12. China	10	1.6	50.0	.125	.172

mean F_{ST} values were considerably high (0.339), and highly significant, for 8 of 12 variable loci, and Wright's F_{IS}, a measure of potential inbreeding within populations (Wright, 1951, 1965), was significantly greater than zero at only one locus, *Ipo* (Table 5). Thus, although each regional population of Korean *K. borealis* appeared to be nearly panmictic, mean F_{ST} for all loci implies that 34% of the total genetic variance is distributed among populations within Korean *K. borealis*, and *Nm* for Korean *K. borealis* was considerably low (0.487).

Finally, Wright's F-statistics showed that populations of Korean *K. borealis* exhibited highly significant allele-frequency heterogeneities among populations at almost all variable loci (Table 5) and it means that there are significantly low levels of gene flow (F_{ST}=0.339, *Nm*= 0.487) among the Korean *K. borealis* populations.

Discussion

Since the patterns of genetic variation reflect the population structure of a species, which can influence the likely mechanisms of divergence and speciation, the studies of genetic variation for amphibian species are useful to understand the evolution and conservation of the species.

In this study, we performed allozyme analysis on Korean *K. borealis* populations to assess the genetic diversity and population structure. In comparison with other Korean amphibian species (see Table 6), Korean

Table 5. Summary of F-statistics at the 12 variable loci found within the Korean *Kaloula borealis* populations

Locus	F_{IS}[a]	F_{IT}	F_{ST}[b]
aGpd	-.055	-.005	.047
Idh-1	.138	.247	.127***
Pgm	-.036	.170	.199***
Mdh-1	-.055	-.005	.047
Mdh-2	-.018	-.002	.016
Sdh	-.145	.021	.145***
Pept	.094	.264	.188***
Est-2	-.022	-.002	.020
Est-3	.001	.096	.095***
Mpi	.059	.458	.424***
Ipo	.156*	.416	.308***
6Pgd	-.010	.549	.554***
Mean	.035	.362	.339

Significance levels for chi-square tests H_0: F_{IS}=0, and H_0: F_{ST}=0; *P<0.05; **P<0.01; ***P<0.001.
[a] Chi-square=$F_{IS}^2 N(k\text{-}1)$, df=$[k(k\text{-}1)]/2$ (Waples, 1987), where N is the total number of individuals and k is the number of alleles at each locus. [b] Chi-square=$2NF_{ST}(k\text{-}1)$, df=$(k\text{-}1)(s\text{-}1)$ (Waples, 1987), where N and k are given above, and s is the total number of populations sampled.

K. borealis has low levels of genetic diversity (He= 0.045) and gene (*Nm*=0.487) among regional populations, in spite of moderate genetic differentiation among populations (Nei's D=0.020).

According to Soule (1976), three conditions (large, old, and slow evolving) must be met before a population can have very high levels of variability. In the theory, population size is based on density, geographic range, and vagility, and then, population age refers to the number of generations since the last bottleneck. In Korea, the geographic range (Distribution: Table 6) and the probable population age (Nei's D: Table 6) of Korean *K. borealis* are moderate in comparison with other Korean amphibian species studied (Fig. 1, Table 6), whereas the population size, based on density, of this species is distinctly being decreased and the distribution is heavily patched because of developmental destruction and chemical pollution of natural habitats, marshes, and rice fields, in and around the village.

Throughout this paper, F_{ST} was used as an estimate of gene flow. In view of the inherent inaccuracies in estimating *Nm* (Slatkin and Barton, 1989; Whitlock, 1992), F_{ST} values were used as a qualitative indication of the magnitude of gene flow. Following Porter's (1990) general guide: F_{ST}<0.2 (*Nm*>1), gene flow is

Table 4. Rogers' (1972) genetic similarity coefficients (S; above diagonal) and Nei's (1972) distance (D; below diagonal) based on allele frequencies of 28 genetic loci among 12 populations of *Kaloula borealis* in Korea and China

Population	1	2	3	4	5	6	7	8	9	10	11	12
1. Inchon	-	.956	.976	.956	.941	.939	.949	.952	.935	.921	.959	.894
2. Pochon	.020	-	.955	.978	.960	.962	.987	.985	.921	.921	.979	.887
3. Chochiwon	.005	.024	-	.958	.944	.950	.949	.941	.945	.933	.949	.914
4. Kwangju	.020	.004	.021	-	.973	.971	.966	.970	.920	.924	.962	.895
5. Wanju	.027	.017	.027	.008	-	.964	.947	.953	.909	.916	.947	.881
6. Yongam	.032	.017	.023	.010	.016	-	.949	.958	.943	.948	.950	.909
7. Sangju	.021	.002	.025	.009	.030	.021	-	.988	.930	.913	.988	.884
8. Kyongju	.023	.003	.030	.008	.028	.019	.002	-	.936	.915	.989	.872
9. Andong	.015	.004	.021	.010	.030	.021	.002	.002	-	.939	.923	.882
10. Bukcheju	.041	.055	.024	.049	.065	.024	.051	.051	.044	-	.947	.903
11. Namcheju	.032	.036	.019	.029	.036	.013	.037	.037	.033	.011	-	.882
12. China	.037	.044	.019	.038	.045	.026	.044	.053	.045	.024	.021	-

Table 6. The average degrees of genetic diversity (He), genetic differentiation (Nei's D), and gene flow (F_{ST}, *Nm*) of regional populations in Korean amphibian species

Species	No. of populations	No. of loci	He	Nei's D	F_{ST} (*Nm*)	Distribution
Rana nigromaculata	24	28	0.043	0.008	0.149 (1.427)	over all
R. plancyi	4	28	0.048	0.007	0.096 (2.354)	western
R. dybowskii	19	18	0.124	0.012	0.081 (2.836)	over all
R. huanrenensis	10	18	0.067	0.008	0.092 (2.467)	northern
R. amurensis	12	18	0.086	0.027	0.237 (0.805)	over all
R. rugosa	28	22	0.087	0.050	0.338 (0.490)	over all (2 genetic groups)
Kaloula borealis	11	28	0.045	0.020	0.339 (0.487)	over all
Bufo gargarizans	9	24	0.058	0.004	0.106 (2.108)	over all (except southern)
B. stejnegeri	9	28	0.083	0.010	0.116 (1.905)	northern
Hyla japonica	35	28	0.121	0.020	0.125 (1.750)	over all
H. suweonensis	3	28	0.047	0.007	0.091 (2.497)	northwestern

moderate (important in promoting genetic similarity); $0.2<F_{ST}<0.33$ ($0.5<Nm<1$), gene flow is weak, but would permit exchange of alleles; $F_{ST}>0.33$ ($Nm<0.5$), gene flow is unimportant and populations are more or less completely isolated. In this study, although Nei's average genetic distance coefficients (D=0.020) indicate no significant genetic differentiation among regional populations in Korean *K. borealis*, the level of genetic heterogeneities found among Korean *K. borealis* populations were considerably high (F_{ST}=0.339), relative to the values within the other Korean frog species studied and the observed levels of gene flow among regional populations of this species (Nm=0.487) is distinctly very low in comparison with other Korean amphibian species studied (Table 6). In this study, the distinctly low level of gene flow is probably due to the results of patchy distribution and low migration range of *K. borealis* in Korea. In thus subdivided populations, the loss of genetic variability can be a consequence of selection or genetic drift because that these can lead to the fixation of alleles at all loci when there is no gene flow (Mayr, 1970; Soulé and Yang, 1974; Lewontin, 1974; Gorman et al., 1975; Berry, 1986: Slatkin, 1987; Vida, 1994; Gray, 1996). Therefore, low levels of genetic diversity and gene flow in the Korean *K. borealis* populations are probably due to the results of decreasing population size and patchy distribution of this species.

Historically, the avoidance of inbreeding and the maintenance of genetic variation were considered as the main issues (Loeschcke, et al., 1994). According to Porter's (1990) general guide, the degree of gene flow among regional populations of Korean *K. borealis* ($F_{ST}>0.33$, $Nm<0.5$) means that gene flow is unimportant in promoting genetic similarity and populations are more or less completely isolated. The absence of gene flow among regional populations of this species is an increase in the amount of mating between relatives within only one patchy population, and hence inbreeding within the species. Moreover, the future of species diversity is in the genetic diversities of the species (Vida, 1994). In general, the higher the maintained genetic diversity, the higher the adaptability and, consequently, the survival probability of species in a changing world. In practice, the Ulreung island population of Korean *R. nigromaculata* which had a small population size and probably met with the allele fixation by the impact of genetic drift, colonized from several mainland populations in the 1920's, is extinct in our 1997 surveys (Yang et al., 1999).

Finally, the low level of genetic diversity and the patchy distribution of Korean *K. borealis* can lead to the extinction of the species from Korea in the near future.

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Appendix I. Buffer systems and enzymes for the analysis of horizontal starch gel electrophoresis of *Kaloula borealis* in this study

Buffer system	E. C. No.*	Enzyme	Condition
Continuous tris citrate II (pH 8.0)	1.1.99.5	α-Glycerolphosphate dehydrogenase (*αGpd*)	100 V/3hrs
	1.1.1.42	Isocitrate dehydrogenase (*Idh-1,2*)	
	2.7.5.1	Phosphoglucomutase (*Pgm*)	
	1.4.1.2	Glutamate dehydrogenase (*Gdh*)	
	1.1.1.37	Malate dehydrogenase (*Mdh-2*)	
	1.1.1.14	Sorbitol dehydrogenase (*Sdh*)	
	3.4.11.1	Leucine amino-peptidase (*Lap*)	
	2.7.3.2	Creatine kinase (*Ck*)	
LiOH (pH 8.1)	5.3.1.9	Phosphoglucose isomerase *(Pgi)*	250 V/3hrs
	3.1.1.1	Esterases *(Est-1,2,3)*	
	N. S.**	General protein (*Gp-1,2,3*)	
	2.6.1.1	Glutamate oxaloacetate isomerase (*Got-1,2*)	
	3.4.11.11	Peptidase (*Pept*)	
Discontinuous tris citrate (pH 8.2)	4.2.1.3	Aconitase (*Aco*)	200 V/3hrs
	1.1.1.37	Malate dehydrogenase (*Mdh-1*)	
	1.15.1.1	Indophenol oxidase (*Ipo*)	
	1.1.1.27	Lactate dehydrogenase (*Ldh-1,2*)	
	5.3.1.8	Mannose-6-phosphate isomerase (*Mpi*)	
Tris maleic EDTA (pH 7.4)	1.1.1.204	Xanthine dehydrogenase (*Xdh-1,2*)	100 V/4hrs
	1.1.1.44	6-Phophogluconate dehydrogenase (*6Pgd*)	
	1.1.1.40	Malic enzyme (*Me-1,2*)	

* Enzyme commisson number, ** Non specific