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ANALISIS GENETIK GENUS GUDGEON *Hypseleotris* (Pisces-Eleotridae) DI AUSTRALIA TENGGARA
(Genetic analysis of the gudgeon genus *Hypseleotris* [Pisces-Eleotridae] species complex in Southeastern Australia)

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ABSTRAK

Studi tentang genetika variasi pada tingkat intra dan interpopulasi gudgeon genus *Hypseleotris* (Pisces-Eleotridae) species kompleks telah dilakukan di Australia Tenggara, khususnya populasi ikan yang terdapat didalam perairan tawar Murray-Darling sistem dan sungai didaerah pesisir. Tujuan studi ini adalah melakukan analisa terhadap kekerabatan genetika diantara species dan pendugaan terhadap adanya proses spesiasi pada species kompleks ini. Duapuluh enam enzym dideteksi dari daging ikan dengan menggunakan allozyme elektrophoresis. Analisa variasi terhadap 28 loci untuk 21 ekor ikan dalam 18 enzym menunjukkan adanya perbedaan species secara sympatry dan mereka berbeda species. Analisa dengan beberapa set enzym polimorphic menunjukkan adanya 3 species yang berbeda nyata. Namun allel frekwensinya berbeda dalam populasi, namun tidak mengikuti pola hukum keseimbangan Hardy-Weinberg. Dengan kisaran inbreeding coeficient diantara 0.03 dan 0.70 pada *Hypseleotris* B1 dan B2, serta nilai relatif nul didapatkan pada *Hypseleotris* A. Analisa genetika menunjukkan bahwa *H. compressa* dan *H. klunzingeri* adalah 'sister species' yang terpisahkan oleh dataran tinggi bagian timur benua Australia. Hal tersebut juga berlaku pada *H. galii* yang mempunyai kekerabatan dengan *Hypseleotris* B1 dan relatif berjarak dengan *Hypseleotris* A. Struktur populasi *Hypseleotris* adalah "isolation by-distance" dan mungkin "ring species".

Kata Kunci : Gudgeon, *Hypseleotris*, allozymes.

ABSTRACT

This study was carried out on the genetic variation of fish intra and interpopulation in the genus *Hypseleotris* (Eleotridae) in Southeastern Australia, particularly within the Murray-Darling river system. Objectives of this study were to analyze genetic relationships between species and consider the process of speciation in this species complex. Twenty-six enzymes were detected in the muscle tissues using allozymes electrophoresis. Analysis of variation at 28 loci for 21 specimens within 19 enzymes showed that the forms identified were maintaining fixed differences in sympatry and were different species. Analysis of the latter data set showed significant differences in allele frequencies between populations and that most populations were out of Hardy-Weinberg Equilibrium with inbreeding coefficients commonly between 0.03 and 0.70 in forms B1 and B2, but not in form A. Genetic results show that *H. compressa* and *H. klunzingeri* are sister species, primarily separated by the eastern uplands.The other coastal species, *H. galii* is related to *H.* form B1 and, distantly, to *H.* form A. Population structure of *Hypseleotris* follows an isolation by-distance model and there may be ring species.

Key words: Gudgeon, *Hypseleotris*, Allozymes.

INTRODUCTION

Members of the genus *Hypseleotris* are small fish found in shallow water, 10-25 cm deep, in aquatic vegetation or under snags along the edges of rivers.

The species of *Hypseleotris* eat crustaceans, mosquito larvae and algae (Lake, 1967; Hoese, et.al., 1980; Grant, 1982). According to Scott, et.al., (1974), juveniles of *H. klunzingeri* are the main food of young Murray cod (*Maccullochella peeli*). Because of their place in the food chain, western carp gudgeons are a key part of the food supply of Murray cod in the inland rivers.

Clearly, the nature and causes of the very high level of variation in form seen in *H. klunzingeri sensu lato* in the inland rivers is of interest to our systematic and ecological understanding of freshwater fish in Australia. The study of genetic variation in this group may reveal whether *H. klunzingeri sensu lato* is one or many species and their relationships to one another. Similarly, the

examination of relationships amongst forms in southern Queensland may well allow the relationships between inland and coastal forms to be clarified.

Genetic Structure of Fish Populations and *Hypseleotris*

Fish populations often maintain different allele frequencies in different parts of their range. This subdivision into subpopulations is not complete however, as sufficient gene flow occurs to maintain the conesion of the overall gene pool. Research on the issues may use DNA sequencing, allozyme electrophoresis, and mitochondrial DNA methods (e.g., RFLP) for analysis of population structuring and differentiation (Richardson, et.al., 1986; May and Krueger, 1990; Avise, 1994; Slatkin, 1994).

There are many factors which affect the level of gene flow between sub-populations including, geographical distance, separation into different habitats or other behavioural differences.

Geographical barriers acting as isolating mechanisms are a fact of evolution. For example *Hypseleotris* comes from a marine ancestor, but now can be found in coastal and inland rivers. There may be relationships between populations of coastal fish and inland fish in southeastern Australia. *H. compressa* and *H. galii* live in coastal rivers and *H. klunzingeri sensu lato* in coastal and inland rivers.

Studies of the genetic structure of other groups of freshwater fish in southeastern Australia have shown the significance of geographical barriers within river systems and the strong isolating effect of the Eastern Highlands. Populations of the Golden Perch (*Macquaria ambigua*), and the Crimson-spotted Rainbowfish (*Melanotaenia fluviatilis* and *M. duboulayi*), show considerable genetic divergence between different localities in the Murray-Darling river system. As well, the genetic distance between populations of the Golden Perch within the Murray-Darling river system and the coastal rivers show the level of differentiation expected at the subspecies level, but there are no morphological differences observed between the two populations (Musyl and Keenan, 1992).

On the basis of the results reported for other fish species in southeastern Australia. It could be predicted that genetic structuring would be present in *Hypseleotris* and that there may be little or much correlation in the patterns obtained using allozyme data sets.

MATERIALS AND METHODS

Sample sets of fish were collected from various localities in the Murray-Darling River system and from coastal rivers in southeastern Australia. The sites were chosen to cover the range of habitats and systems inhabited by the complex. Fish were collected between December 1995 and December 1998 in each summer season.

Genetic analysis of the fishes was carried out using allozyme electrophoresis of the muscle tissue sample collected from each fish. All samples were sorted by morphological form and location for allozyme electrophoresis. Twenty-six enzymes were detectable using muscle samples. The zymograms were interpreted based on the resolution of bands obtained on cellulose acetate gels after staining for specific enzymes. The gels were stained using the procedures in Richardson, et al., (1986).

A small piece of sample tissue was placed in microcentrifuge tubes and blended by hand into a paste with a small amount of cold lysing solution, and spun at 10.000 g at 4^0C in a microcentrifuge for 7 minutes. The supernatant was applied to the gel for electrophoresis assay.

Allozyme variation of *Hypseleotris* was examined using three buffer systems: 0.015 M Tris-borate (TEB) pH 7.8 and 0.01 M Tris-Maleate (TM) pH 7.8, both of buffer solutions were run at 150 V for one hour. The other buffer was 0.01 M Citrate Phosphate (CP) pH 6.4, it was run at 100 V for one hour electrophoresis. Most procedures were carried out in a cool room (4^0 C) and are modifications of Richardson, et al, (1986), Shaklee and Salini (1985). Then the standard nomenclature of protrein coding loci was used, following Shaklee, et.al., (1990).

Statistical Analysis

The genotypic data was analysed to find consistently differing forms in the *Hypseleotris* species complex.

Analysis of genetic data was carried out using the BIOSYS-1 program, Release 1.7 (Swofford and Selander, 1989). The percentage of polymorphic loci and the mean expected heterozygosity across loci were measured for genetic variability. Analysis of genetic differentiation among of population was carried out using variation in gene frequencies and inbreeding coefficient (Wright, 1978). Then, genetic similarities and distances were measured using Roger's genetic similarity and distance (Roger, 1972), and unbiased genetic distance (Nei, 1978). Phenetic relationships amongst populations

were analysed by cluster analysis using Unweighted Pair Group Method Analysis (UPGMA), (Sneath and Sokal, 1973). The network of populations was analysed using the unrooted Distance Wagner method.

RESULT AND DISCUSSION

Survey for Genetic Variation

To test the taxonomic hypotheses regarding *Hypseleotris* developed using morphological data, 21 individuals, several from each form of *Hypseleotris* identified, were analyzed for genetic variation and this variation used to determine genetic relationships between forms.

Twenty-six enzymes were surveyed, only eighteen enzymes showing sufficient activity for study (ADA, ADH, FUM, PGM, LDH, 6PGD, GOT, GPI, MDH, ENOL, ME, IDH, EST A, EST B, PEPB, GPT, PGAM, CA, GPD). The enzymes surveyed included hydrolases, isomerases, lyases, oxidoreductases and transferases. The electrophoresis results are summarized in Table 1., where putative homozygote and heterozygote phenotypes are shown. The genetic survey of enzymes includes the products of 28 loci and included 4 monomeric, 11 dimeric and 3 tetrameric enzymes.

Five of the enzymes (6PGD, MDH-2, ENOL-1, ENOL-2 and IDH-1) were monomorphic for all samples studied. Only the variable loci were used to examine population structure within and between populations of *Hypseleotris*.

Principal component analysis using all alleles as characters, with values of 1 for homozygotes, and 0.5 for heterozygotes, were carried out. A total of 65 % of the variation was explained by principal components 1 to 4.

In figure 1, the scattergram using the first and second principal components shows the specimens separated distinctly into five groups, *H. compressa*, *H. galii*, *Hypseleotris* A, *Hypseleotris* B1, *Hypseleotris* B2. Forms *Hypseleotris* A, *Hypseleotris* B2 are clearly separated from the remaining forms.

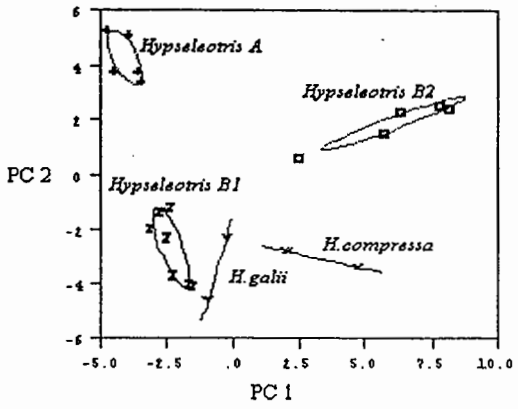

Figure 1. Scatterplot of coastal and inland *Hypseleotris*, based on the first principal component, PC 1 (26 %) and second principal component, PC 2 (16 %), bivariate normal ellipse p=0.50.

The scatterplot between PC 1 and PC 2 apparently places *H. galii* close to *Hypseleotris* form B1. However, these fish actually were not close, because *H. galii* and *Hypseleotris* B1 were separated by principal component 4. The reverse situation is true for *H. compressa* and *Hypseleotris* form B2, which are more clearly separated by principal component 2.

It is noteworthy that the twenty-one fish studied, though taken from widely different localities still clustered in groups reflecting the forms found in the morphological analysis. The average heterozygosity per locus among the 26 loci in each form was calculated (see Table 2).

Table 2. Genetic Variability of *Hypseleotris* at 26 loci in all populations

	H. compressa	*H. galii*	*H. form A*	*H. form B1*	*H. form B2*
Mean H direct count (D.C.)	0.184	0.129	0.197	0.132	0.201
Standard Error (S.E.)	0.066	0.051	0.048	0.042	0.055
Mean no. of alleles per locus	1.7	1.7	2.3	2.2	2.2
Standard Error (S.E.)	0.2	0.2	0.2	0.2	0.2
Percentage of loci polymorphic	42	46	69	58	62
Mean sample size per locus	19(±6)	17(±5)	72(±17)	94(±22)	44(±11)

The observed mean heterozygosity (direct count) per locus varied among forms (Table 2) with the morphologically similar forms, *H. galii* and *H.* form B1 having lower levels of variation than the remainder. The mean heterozygosity values obtained were higher than found for other freshwater fish in the Murray-Darling River system, such as the golden perch (*Macquaria ambigua*) 0.036 ± 0.017 (Musyl and Keenan, 1992), or the coastal catadromous percoid Australian bass (*Macquaria novemaculeata*) 0.043 ± 0.013 (Jerry, 1997). It was also greater than that found using a wide range of fish species (0.051 ± 0.03) (Nevo, 1977).

Number of species of *Hypseleotris*

The allozymes electrophoresis data shown in Table 1 were used to examine the genetic relationships between the different morphological forms. Two measures were used, namely Nei's unbiased genetic distance (Nei, 1978) and Roger's genetic similarity (Rogers, 1972). The results are summarized in Table 3.

Table 3. Matrix of genetic similarity and/or distance coefficients

	H. compressa	*H. galii*	*H. form B2*	*H. form B1*	*H. form A*
H. compressa	********	0.255	0.151	0.295	0.372
H. galii	0.678	********	0.288	0.172	0.223
H. form B2	0.726	0.644	********	0.283	0.321
H. form B1	0.656	0.740	0.663	********	0.171
H. form A	0.607	0.699	0.635	0.757	********

Below diagonal : Roger's genetic similarity (Roger, 1972)
Above diagonal : Nei's unbiased genetic distance (Nei, 1978)

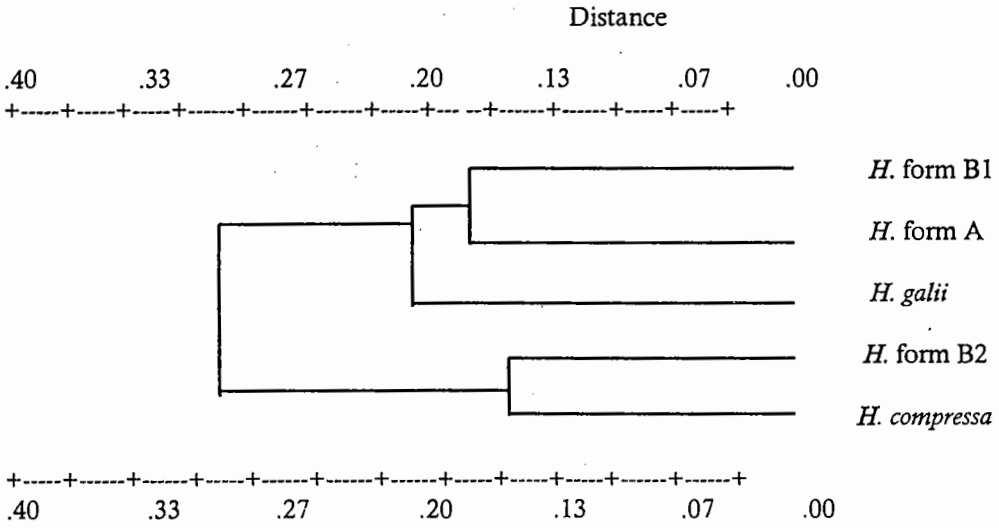

Figure 4.3. Cluster of *Hypseleotris* based on UPGMA dendogram plot upon unbiased genetic distance (Nei, 1978).

The genetic distance of *Hypseleotris* between the two recognized species in the complex (*H. compressa* and *H. galii*) is 0.255. *H.* form A has a divergence value greater than this from all forms except *H.* form B1. However, *H.* form B1 is also clearly separated from *H. compressa* and *H.* form B2. *H.* form B2 is also well separated from all forms except *H. compressa*. The comparatively large divergence values obtained and the principal component analysis (PCA, Figure 4.2) would support the view that all five forms studied are separate species.

Cluster analysis was used to identify relationships between the forms using the unweighted pair group method analysis (UPGMA, Sneath and Sokal, 1973) and the coefficients of Nei's unbiased genetic distance as the data set, (Table 4.5).

The result is shown in Figure 4.3, two groups (*H. galii*, *H.* form B1, *H.* form A and *H. compressa* , *H.* form B2) were found in the analysis.

Population Structure of *Hypseleotris* in the Southeastern Australia

Populations of *Hypseleotris* form B2 were obtained from two river systems, and the results are summarized in Table 3. Fish population at Leslie Dam (Warwick), Albert Priest Channel (Canal), and Burrendong Dam have a relatively similar inbreeding coefficients (F) 0.33 - 0.50. Such results are quite surprising for a natural population and are what would be expected of the samples sets consisted sib and cousins (Mettler and Gregg, 1969). The inbreeding coefficient for the population of *H.* form B2 from Forbes is 0.17 (half-sibs) and the Nyngan population has an inbreeding coefficient (-0.05) close to 0 (zero), see Table 4.

In a similar fashion, the inbreeding coefficients obtained for populations of *H.* form B1 from the Murray-Darling River system show that these populations of fish are highly inbreed (equivalent to sib-parents), except again the population of fish from Narrandera (0.03), see Table 5.

Table 4. Genetic Variability of *Hypseleotris* form B2 at seven loci in all populations

					Mean heterozygosity	
Population	*2N*	Mean no. of alleles perlocus	Percentage of loci polymorphic	Inbreeding Coeficient (F)	Direct count	HW expected
Warwick	54	2.3±0.4	57.1	0.36	0.14±0.08	0.22±0.09
Bourke	12	1.4±0.2	42.9	nc	nc	nc
Nyngan	24	1.7±0.3	57.1	-0.05	0.20±0.11	0.19±0.08
Canal	28	2.4±0.3	71.4	0.50	0.16±0.05	0.32±0.10
Burrendong Dam	90	2.1±0.4	71.4	0.45	0.13±0.07	0.24±0.08
Forbes	22	2.3±0.3	85.7	0.17	0.29±0.14	0.35±0.10
Narrandera	16	2.3±0.2	100.0	nc	nc	nc
Albury	10	1.7±0.3	57.1	nc	nc	nc

nc: not calculated, because the samples number was low.

Table 5. Genetic Variability of *Hypseleotris* form B1 at seven loci in all populations

					Mean heterozygosity	
Population	*2N*	Mean no. of alleles per locus	Percentage of loci polymorphic	Inbreeding Coeficient (F)	Direct count	HW expected
Warwick	46	2.6±0.5	71.4	0.21	0.19±0.12	0.24±0.09
Nyngan	38	2.0±0.3	57.1	0.52	0.12±0.07	0.25±0.09
Canal	26	1.9±0.3	57.1	0.31	0.20±0.10	0.29±0.11
Lake Windamere	58	2.1±0.3	42.9	0.57	0.06±0.03	0.14±0.06
Sofala	60	1.7±0.3	28.6	0.27	0.11±0.07	0.15±0.09
Forbes	70	2.4±0.5	57.1	0.68	0.07±0.03	0.22±0.08
Narrandera	70	2.0±0.4	57.1	0.03	0.31±0.13	0.30±0.11
Albury	20	1.6±0.3	42.9	0.70	0.06±0.04	0.20±0.10

The calculation of inbreeding coefficients was made for *Hypseleotris* form A in each of four populations in the Murray-Darling River system and the results are shown in Table 6.

The population of *H.* form A of Warwick has a low inbreeding coefficient (-0.03). While the inbreeding coefficients of fish populations from the Murray River system i.e. Forbes (Lachlan drainage) is -0.23, Narrandera (Murrumbidgee drainage) is -0.41 and Albury (Murray drainage) is -0.35. The result is due to the presence of too many heterozygotes at all loci studied. However, the Confidence Intervals (CI) are very large and the results may not differ significantly from 0 (zero).

Table 6. Genetic Variability of *Hypseleotris* form A at seven loci in all populations

Population	*2N*	Mean no. of alleles per locus	Percentage of loci polymorphic	Inbreeding Coeficient (F)	Mean heterozygosity: Direct count	Mean heterozygosity: HW expected
Warwick	70	2.0±0.4	57.1	-0.03	0.32±0.16	0.31±0.11
Bourke	8	1.3±0.2	28.6		nc	nc
Nyngan	16	1.9±0.3	71.4		nc	nc
Canal	18	1.9±0.3	71.4		nc	nc
Lake Windamere	8	2.1±0.3	85.7		nc	nc
Sofala	8	1.6±0.2	57.1		nc	nc
Forbes	74	2.4±0.4	71.4	-0.23	0.32±0.17	0.26±0.08
Narrandera	60	1.9±0.3	57.1	-0.41	0.38±0.17	0.27±0.10
Albury	32	2.3±0.3	57.1	-0.35	0.42±0.15	0.31±0.01

To examine these abnormal genotype distributions further, data was analyzed from 17 loci (ADA, PGM, LDH, GOT-1, GOT-2, GPI-1, GPI-2, MDH-1, MDH-2 ME-1, ME-2, ME-3, IDH-1, IDH-2, PEPB-1, PEPB-2 and CA-1) in the three forms from two localities within the Macquarie River system, Lake Windamere and the Little River tributary of the Macquarie River.

The inbreeding coefficients of *H.* form B1 and *H.* form A are significantly different, *H.* form B1 from the Little River has on F value 0.43 and 0.22 for Lake Windamere fish. While *H.* form A has an inbreeding coefficient value 0.00 for Little River population and 0.12 at Lake Windamere, see Table 7. The data for *H.* form B1 these is similar to that found previously. The *H.* form A data however does not show the excess of heterozygotes seen elsewhere.

Table 7. Genetic Variability of *Hypseleotris* at 17 loci in the Macquarie River System, at Lake Windamere (LW) and Little River (LR).

Population	*2N*	Mean no. of alleles per locus	Percentage of loci polymorphic	Inbreeding Coeficient (F)	Mean heterozygosity: Direct count	Mean heterozygosity: HW expected
H. B1 (LR)	24	1.8±0.2	52.9	0.43	0.13±0.06	0.23±0.06
H. A (LR)	40	2.0±0.2	64.7	0.00	0.27±0.08	0.27±0.06
H. B2 (LR)	20	1.9±0.2	58.8	-0.15	0.31±0.11	0.27±0.07
H. B1 (LW)	52	1.8±0.2	58.8	0.22	0.14±0.05	0.18±0.04
H. A (LW)	30	1.8±0.2	58.8	0.12	0.22±0.08	0.25±0.06

The alternate hypotheses of several species in each form was examined by attempting to identify linkage disequilibrium between loci. That is patterns of double homozygotes for alternate alleles were sought and none were found. Consequently no genetic evidence of more than

one species in each form was obtained and other explanations for the high inbreeding coefficients and interpopulation changes in morphology and alelle frequencies are needed.

Comparison of Sympatric Populations of Inland *Hypseleotris* forms

To further test the specific status of the morphological forms of *Hypseleotris* detected in the Murray-Darling River system, sympatric populations of the forms were studied to determine whether independent gene pools were maintained in sympatry. The principal component analysis of genetic characters from populations at two locations were used based on 18 loci within 10 enzymes (ADA, PGM, LDH, GOT-1 and 2, GPI-1 and 2, MDH-1 and 2, ME 1,2, and 3, IDH-1 and 2, PEPB-1 and 2, CA-1 and 2). The sympatric populations of fish from Little River and from Lake Windamere were used.

The Little River is a tributary in the head waters of the Macquarie River system. The scatterplot of specimens from the Little River for PC1 and PC2 for pincipal component analysis are shown the intermediate fish showed that these fish were heterozygotes.

The population of *Hypseleotris* in Lake Windamere about 100 Km from Little River was sampled during each summer season between 1996 and 1998. A scatterplot of PC 1 and PC 2 values calculated using the Principal Component Analysis the three forms maintain clearly distinguishable gene pools in sympatry.

When the data from the two localities used in the previous section are combined, it can be seen that there are genetic difference between the same forms from different localities for *H.* form A and *H.* form B1. These locations differ in ecological attributes with stream flow very slow in Lake Windamere and regular in the Little River. However, genetic drift may also have influenced these populations of fish and caused the changes between the two localities.

CONCLUSION

Dengan analisis genetika terhadap ikan *Hypseleotris* yang merupakan spesies komplek didalam sistem Sungai Murray-Darling di Australia Tenggara, ditemukan tiga jenis ikan *Hypseleotris* yang dikenal sebagai *Hypseleotris* B1, *Hypseleotris* B2 dan *Hypseleotris* A. Pada umumnya, ikan *Hypseleotris* yang berada di perairan sungai hidup secara simpatrik, dan ikan tersebut mempunyai kekerabatan dengan ikan *Hypseleotris* yang berada di sungai daerah pesisir, seperti *H. compressa* and *H. galii.*

Struktur populasi *Hypseleotris* di Australia Tenggara adalah "isolation by-distance" dan kemungkinan "ring species".

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