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Genetic Differentiation of *Oryzias minutillus* in Thailand

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Abstract An allozyme survey was conducted in eleven local populations of *Oryzias minutillus* from Thailand. The region-specific distribution of alleles and the genetic relationship among the eleven populations revealed that they represented major three population groups, within the country as a whole; the Peninsular, Mae Nam Chao Phraya and Mekong subpopulations. Because their distribution boundaries coincide with geographic features, it is supposed that their genetic differentiation is primarily due to geographic isolation. Karyotype polymorphism has been reported only from the Mae Nam Chao Phraya subpopulation, suggesting that the variant karyotype evolved after allopatric isolation of the three subpopulations.

The genus *Oryzias* is a small freshwater fish, consisting of ten species found in standing waters in South, Southeast and East Asia (Yamamoto, 1975; Labhart, 1978). *O. minutillus* is distributed in Thailand (Smith, 1945; Magtoon and Uwa, 1985; Magtoon, 1986), Burma and Yunnan, southern China (Uwa et al., 1988).

Karyotype polymorphism has been reported in *O. minutillus* from Thailand (Magtoon and Uwa, 1985; Magtoon et al., 1992). The number of chromosomes in this species ranges from 28 to 42, with chromosomes carrying nucleolus organizing regions varying between local populations. It has been suggested that a karyotype with 42 acrocentrics is basic for *O. minutillus* and that reduced chromosome numbers have been derived by pericentric inversions and centric fusions (Ashida and Uwa, 1987; Uwa et al., 1988).

Within the genus *Oryzias*, Sakaizumi (1986), Sakaizumi and Jeon (1987) and Sakaizumi et al. (1980, 1983) have succeeded in determining population structures of *O. latipes* in Japan, China and Korea by allozyme analysis.

In the current study, the genetic features of local *O. minutillus* populations in Thailand were examined using allelic variations of enzymes, and the degree of genetic differentiation among them estimated. This report considers the intraspecific structure of *O. minutillus* related to its geographic distribution and the relationship between genetic differentiation and karyotype polymorphism in the species.

Materials and Methods

O. minutillus were collected in Thailand in October and November, 1989 and August, 1990. The sampling localities and number of electrophoretically examined specimens of *O. minutillus* (in parentheses) are as follows: 1) Chiang Mai (45) and 2) Chiang Rai (45) from the north; 3) Chai Nat (40), 4) Bangkok (45) and 5) Ratchaburi (20) from the central region; 6) Chachoengsao (12) and 7) Rayong (40) form the southeast; 8) Bua Yai (45) and 9) Phayakkhaphum Phisai (9) from the northeast; and 10) Chumphon (45) and 11) Phuket (45) from the south (Fig. 1). Specimens were frozen on dry ice immediately after collection, and stored at -80°C until electrophoretic analysis.

Chiang Mai, Chai Nat, Bangkok, Chachoengsao, Rayong and Ratchaburi belong to the basin of the Mae Nam Chao Phraya River system. Chiang Rai, Bua Yai and Phayakkhaphum Phisai are located within the Mekong River system. Chumphon is located on the slope facing the Gulf of Thailand, and Phuket is an island in the Andaman Sea.

Horizontal starch gel electrophoresis was per-

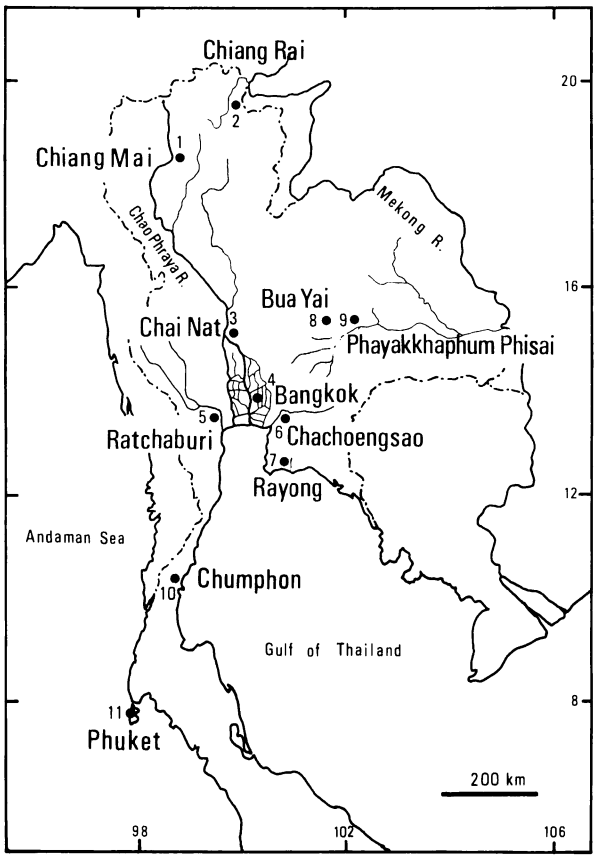

Fig. 1. Collection localities of *Oryzias minutillus* in Thailand.

formed using the four buffer systems described in Clayton and Tretiak (1972), Ridgway et al. (1970) and Shaw and Prasad (1970). Aqueous homogenates of eye balls, liver and muscle samples resolved thirteen enzymes and general proteins encoded by twenty-four loci: creatine kinase (CK; EC 2.7.3.2), esterase (EST; EC 3.1.1.1), fumarate hydratase (FH; EC 4.2.1.2), glucosephosphate isomerase (GPI; EC 5.3.1.9), isocitrate dehydrogenase (IDH; EC 1.1.1.42), lactate dehydrogenase (LDH; EC 1.1.1.27), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), phosphoglucomutase (PGM; EC 2.7.5.1), 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44), Liditol dehydrogenase (IDDH; EC 1.1.1.14), superoxide dismutase (SOD; EC 1.15.1.1) and sarcoplasmic proteins (SP). Staining procedures followed Shaw and Prasad (1970) and Harris and Hopkinson (1976) with slight modifications.

Allelic products were numbered with increasing proportional anodal mobility relative to the origin, using those most common (=100) in the population of *O. minutillus* from Bangkok as standards. The mean heterozygosity over all 24 loci (H) was estimated for each population. Genetic identity and genetic distance values (I and D; Nei, 1972) were calculated from allele frequencies. These genetic data were processed using the BIOSYS-1 computer program of Swofford and Selander (1981).

Results

Twenty-four loci encoding thirteen enzymes and general proteins were assayed. Of these loci, eight (*Ck-3*, *Gpi-1*, *Ldh-3*, *Mdh-2*, *Sod* and *Sp-1*, *-2*, and *-3*) were fixed for the same electromorph in all samples. Electrophoretic variation was observed for the remaining sixteen loci (Table 1).

Locality-specific alleles were observed at several loci. Seven populations, Chiang Mai (locality

Table 1. Allele frequencies for 16 polymorphic loci in 11 populations of *Oryzias minutillus*

Locus	Allele	Population										
		1	2	3	4	5	6	7	8	9	10	11
Ck-1												
	(N)	40	46	40	47	13	11	40	48	9	43	43
	103	—	—	—	.021	—	—	—	.042	—	—	—
	100	.862	1.000	1.000	.862	.538	1.000	1.000	.958	1.000	1.000	1.000
	97	.138	—	—	—	.346	—	—	—	—	—	—
	93	—	—	—	.117	.116	—	—	—	—	—	—
Ck-2												
	(N)	44	42	40	48	20	12	40	48	9	40	45
	127	—	—	—	—	—	—	—	.042	—	—	—
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.958	1.000	1.000	1.000
Est												
	(N)	45	45	40	49	20	12	40	48	9	45	45
	113	.333	—	.075	.071	.075	.042	.013	1.000	1.000	1.000	1.000
	100	.667	.956	.925	.929	.925	.958	.987	—	—	—	—
	98	—	.044	—	—	—	—	—	—	—	—	—
Fh												
	(N)	10	10	40	17	18	12	38	18	9	15	15
	100	.650	1.000	1.000	1.000	.889	1.000	.987	1.000	1.000	1.000	1.000
	74	.350	—	—	—	.111	—	.013	—	—	—	—
Gpi-2												
	(N)	44	45	40	49	20	12	40	48	9	45	45
	125	—	—	.087	—	—	.083	.037	.010	.111	—	—
	112	—	—	.050	—	—	—	.013	—	—	—	—
	100	1.000	1.000	.863	1.000	1.000	.917	.950	.990	.889	1.000	1.000
Gpi-3												
	(N)	44	45	40	49	20	12	40	48	9	45	45
	267	—	—	—	—	—	—	—	.010	.167	—	—
	142	—	—	—	—	—	—	—	.125	.388	—	—
	33	—	—	.038	—	—	—	—	—	—	—	—
	17	—	.044	.138	.245	.025	.292	.213	.615	.167	—	—
	−100	1.000	.956	.824	.735	.950	.708	.774	.146	.278	1.000	1.000
	−130	—	—	—	—	.025	—	.013	—	—	—	—
	−217	—	—	—	.020	—	—	—	.104	—	—	—
Idh-1												
	(N)	44	45	40	49	20	12	40	48	9	45	44
	100	1.000	1.000	1.000	1.000	1.000	.958	1.000	1.000	1.000	1.000	1.000
	90	—	—	—	—	—	.042	—	—	—	—	—
Idh-2												
	(N)	44	45	40	49	20	12	40	48	9	45	45
	118	—	—	—	—	.075	—	—	—	—	—	—
	100	1.000	1.000	.987	1.000	.925	1.000	.987	1.000	1.000	1.000	1.000
	73	—	—	.013	—	—	—	.013	—	—	—	—
Ldh-1												
	(N)	45	46	40	49	20	12	40	48	9	40	45
	105	—	—	—	—	—	—	.013	—	—	—	—
	100	1.000	1.000	1.000	1.000	1.000	1.000	.987	1.000	1.000	1.000	.989
	95	—	—	—	—	—	—	—	—	—	—	.011
Ldh-2												
	(N)	44	45	40	49	20	12	40	48	9	45	45
	100	1.000	1.000	1.000	.980	1.000	.958	1.000	.750	1.000	1.000	1.000
	84	—	—	—	—	—	—	—	.240	—	—	—
	72	—	—	—	.020	—	.042	—	.010	—	—	—

Table 1. Continued

Locus	Allele	Population										
		1	2	3	4	5	6	7	8	9	10	11
Mdh-1												
	(N)	44	42	39	46	20	12	39	48	9	45	45
	112	—	—	.192	—	—	—	.026	.125	—	—	—
	100	.761	.905	.808	.815	.475	.625	.743	.375	.278	1.000	1.000
	97	—	.095	—	—	—	—	—	—	—	—	—
	88	.239	—	—	.185	.525	.375	.231	.500	.722	—	—
Mdh-3												
	(N)	44	45	40	49	20	12	40	48	9	45	45
	130	—	—	—	—	.025	—	—	—	—	—	—
	100	1.000	1.000	1.000	1.000	.975	1.000	1.000	1.000	1.000	1.000	1.000
Me												
	(N)	44	45	40	48	20	12	40	48	9	45	45
	127	—	—	—	.021	—	—	—	—	—	—	—
	114	.023	.011	—	.063	.025	.042	—	—	—	.056	—
	100	.977	.989	1.000	.916	.975	.958	1.000	.406	.610	.944	.944
	95	—	—	—	—	—	—	—	.469	.167	—	—
	82	—	—	—	—	—	—	—	—	—	—	.056
	77	—	—	—	—	—	—	—	.125	.167	—	—
	71	—	—	—	—	—	—	—	—	.056	—	—
Pgm												
	(N)	44	45	40	49	20	12	40	48	9	45	42
	113	—	—	—	—	—	—	—	—	—	1.000	.643
	100	1.000	1.000	1.000	1.000	1.000	1.000	.975	1.000	.944	—	.357
	92	—	—	—	—	—	—	.025	—	.056	—	—
6Pgd												
	(N)	45	45	40	49	20	12	40	48	9	44	45
	100	1.000	1.000	1.000	1.000	1.000	.958	1.000	1.000	1.000	1.000	1.000
	90	—	—	—	—	—	.042	—	—	—	—	—
Iddh												
	(N)	17	13	27	21	20	11	31	44	9	26	37
	230	—	—	—	—	—	—	—	—	.111	—	—
	170	—	—	—	.024	—	—	.016	.068	.278	—	—
	100	.765	.846	.796	.595	.425	.500	.532	.920	.611	1.000	1.000
	30	.235	.154	.204	.381	.575	.500	.452	.012	—	—	—
H		0.081	0.027	0.057	0.075	0.096	0.084	0.062	0.104	0.111	0.004	0.025

1: Chiang Mai, 2: Chiang Rai, 3: Chai Nat, 4: Bangkok, 5: Ratchaburi, 6: Chachoengsao, 7: Rayong, 8: Bua Yai, 9: Phayakkhaphum Phisai, 10: Chumphon and 11: Phuket

number 1) and Chiang Rai (2) in the north, Chai Nat (3), Ratchaburi (5) and Bangkok (4) in the central region, and Rayong (7) and Chachoengsao (6) in the southeast all had the *100* allele as a predominant allele at the esterase locus (*Est*, Fig. 2). All specimens from Chumphon (10) and Phuket (11) in the south, and Bua Yai (8) and Phayakkhaphum Phisai (9) in the northeast, possessed the *113* allele. The two populations in the south had a unique allele at the phosphoglucomutase locus (*Pgm*, Fig. 3). Chumphon (10) had the *113* allele exclusively, and Phuket (11) had this as a major allele. The other nine populations had the *100* allele instead. The glucosephosphate isomerase locus (*Gpi-3*) showed high polymorphism in the northeast populations (Fig. 4). The populations in Bua Yai (8) and Phayakkhaphum Phisai (9) in the northeast had the *17* and *142* alleles as major alleles at the *Gpi-3* locus, respectively. The other nine populations showed high frequency of the *100* allele at that locus. The two populations in the northeast also had locality-specific *95* and *77* alleles at the *Me* locus (Fig. 5). In partic-

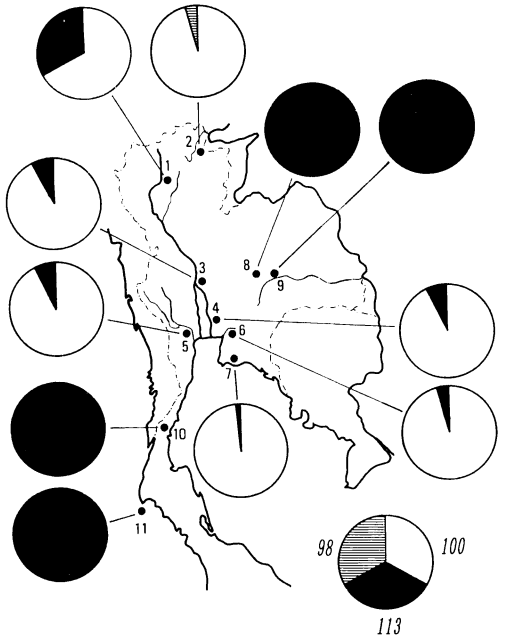

Fig. 2. Geographic distribution of alleles at the esterase locus (*Est*) in *Oryzias minutillus*. See Table 1 for listing of numbers.

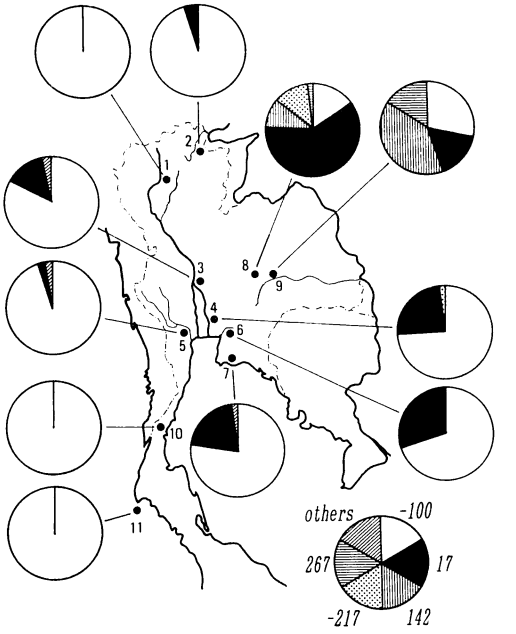

Fig. 4. Geographic distribution of alleles at glucose-phosphate isomerase locus (*Gpi-3*) in *Oryzias minutillus*. See Table 1 for listing of numbers.

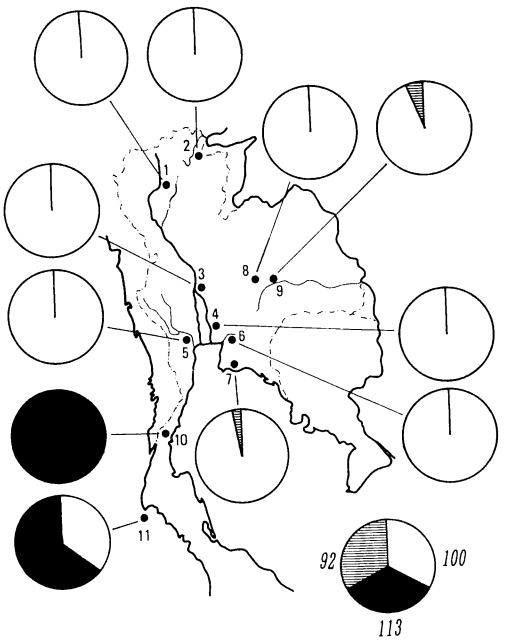

Fig. 3. Geographic distribution of alleles at the phosphoglucomutase locus (*Pgm*) in *Oryzias minutillus*. See Table 1 for listing of numbers.

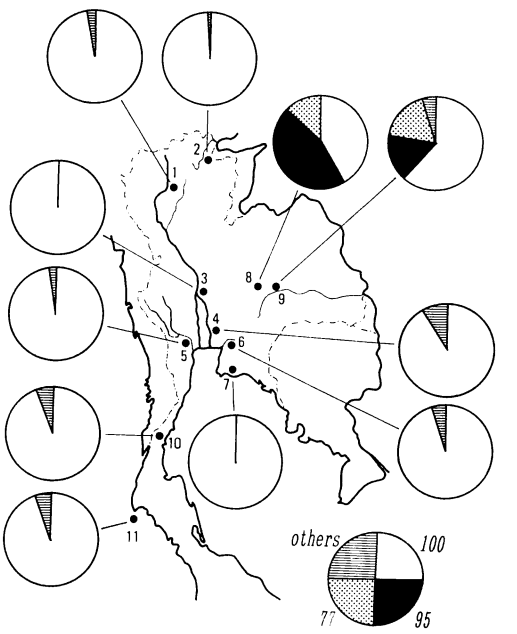

Fig. 5. Geographic distribution of the alleles at the malic enzyme locus (*Me*) in *Oryzias minutillus*. See Table 1 for listing of numbers.

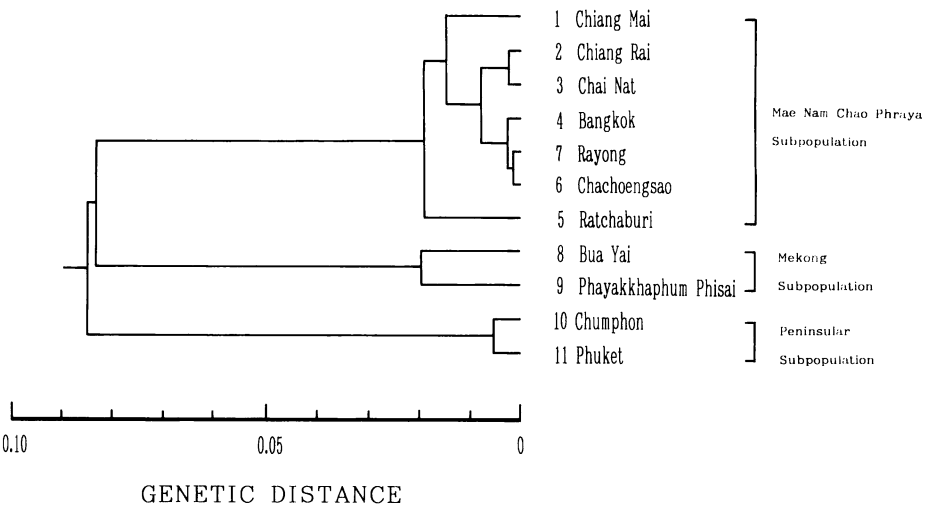

Fig. 6. UPGMA dendrogram derived from genetic distance (Nei, 1972) among 11 populations of *Oryzias minutillus* in Thailand.

ular, the *95* allele was predominant in Bua Yai (8).

Based on combinations of the locality-specific alleles, these results indicated that the over all population of *O. minutillus* in Thailand could be divided into three major subpopulations; Peninsular, Mae Nam Chao Phraya and Mekong. The Peninsular subpopulation, consisting of Chumphon (10) and Phuket (11), could be distinguished from the other local populations by the *113* allele at the *Pgm* locus. The local populations at Bua Yai (8) and Phayakkhaphum Phisai (9), in the northeast, were characterized by the *95* and *77* alleles at the *Me* locus, and were designated as the Mekong subpopulation. The other seven local populations in the north, the central region and the southeast possessed the *100* allele as a major allele at the *Est* locus, and were designated as the Mae Nam Chao Phraya subpopulation.

Genetic identity and distance (Nei, 1972) were calculated for all pairs of local populations (Table 2). The eleven populations, which were clustered using the UPGMA method for D values, fell into three discrete clusters; the Peninsular, Mae Nam Chao Phraya and Mekong subpopulations (Fig. 6). These subpopulations so recognized, coincided exactly with those characterized by locality-specific alleles. The genetic distance among the three subpopulations exceeded 0.08. The Chiang Rai population (2) in the north was included in the Mae Nam Chao Phraya subpopulation on the basis of its locality-specific allele and UPGMA clustering. This population is located in one of the tributaries of the Mekong River system as opposed to the Mae Nam

Table 2. Estimates of genetic distance (above diagonal) and identity (below diagonal) (Nei, 1972) among 11 populations of *Oryzias minutillus* based on 24 loci

Population	1	2	3	4	5	6	7	8	9	10	11
1 Chiang Mai	—	.013	.013	.014	.019	.019	.015	.078	.065	.076	.050
2 Chiang Rai	.988	—	.003	.006	.027	.013	.007	.098	.090	.088	.062
3 Chai Nat	.987	.997	—	.005	.026	.010	.005	.086	.080	.088	.062
4 Bangkok	.986	.994	.995	—	.015	.004	.002	.081	.074	.096	.070
5 Ratchaburi	.981	.974	.975	.985	—	.014	.015	.106	.084	.123	.095
6 Chachoengsao	.981	.987	.990	.996	.986	—	.002	.083	.072	.110	.083
7 Rayong	.985	.993	.995	.998	.986	.998	—	.091	.079	.103	.077
8 Bua Yai	.925	.907	.917	.922	.899	.921	.913	—	.020	.102	.075
9 Phayakkhaphum Phisai	.937	.914	.923	.928	.919	.931	.924	.980	—	.093	.068
10 Chumphon	.927	.916	.915	.908	.885	.896	.902	.903	.911	—	.005
11 Phuket	.951	.940	.940	.932	.909	.920	.926	.928	.935	.995	—

Chao Phraya River system.

Discussion

The region-specific distribution of alleles and the genetic relationships among the eleven local populations of *O. minutillus* indicated the existence of three major subpopulations, Mae Nam Chao Phraya, Mekong, and Peninsular, whose distribution coincided closely with regional geographic features. The habitat ranges of the Mae Nam Chao Phraya and the Mekong subpopulations correspond to the large, local river systems, the Chao Phraya River system and Mekong River system, respectively. The Phang Hoei mountains separate these two subpopulations, while the Bilauktung Mountains run through the root of the Malaya Peninsula between the Mae Nam Chao Phraya and Peninsular subpopulations. These geographical barriers, and region-specific, genetic features among the three subpopulations, suggest that their genetic differentiation is primarily due to geographic isolation.

Furthermore, mean heterozygosity varied among the three subpopulations. Mean heterozygosity values in the Peninsular subpopulation, 0.004 in Chumphon (10) and 0.025 in Phuket (11), were the lowest among the three subpopulations, and reflected less heterozygosity than fishes in general (mean piscine heterozygosity: 0.0513; Nevo, 1978). The Mae Nam Chao Phraya and Mekong subpopulations showed relatively high average heterozygosity (0.069 and 0.108, respectively). The Peninsular subpopulation inhabits the narrow, mountainous part of the Malaya Peninsula (the Kra Isthmus) without the benefit of a large river system, such as the Chao Phraya or the Mekong. As a general rule, reduced gene flow between populations, population bottle necks, rates of evolutional divergence and effective population size may influence the magnitude of heterozygosity (Soule, 1971, 1976; Valentine, 1976; Stoneking et al., 1981). Thus, the over all geography of the area inhabited by the Peninsular subpopulation may have inhibited both the growth of a large population and contact with other populations, either of which would result in less variability within the Peninsular subpopulation.

Magtoon and Uwa (1985) reported the existence of karyotype polymorphism in *O. minutillus*. This was subsequently categorized on the basis of arm number and position of nucleolus organizer regions (NORs) (Magtoon et al., 1992). The genetic relationships among the three subpopulations and their distribution coincided with the polymorphic karyological groupings presented by Magtoon et al. (1992). According to their divisions, the Peninsular and Mekong subpopulations were both categorized as the basic type (NF=42, NORs-A), while the Mae Nam Chao Phraya subpopulation was considered an evolved type (NF=44, NORs-SM).

The local population from Chiang Rai was grouped with the Mae Nam Chao Phraya subpopulation on the basis of both the present genetic analysis and the karyological studies of Magtoon and Uwa (1985) and Magtoon et al. (1992), in spite of its location in one of the tributaries of the Mekong River System. Kottelat (1989) indicated that there had been many changes in the river courses affecting the freshwater fish fauna in Indochina. This suggests that the Chiang Rai population became established in one of the tributaries of the Mekong River system by headwater capture from the Mae Nam Chao Phraya River system, after genetic differentiation of the Mekong and Mae Nam Chao Phraya subpopulations. The relatively small heterozygosity of the Chiang Rai population, compared to other members of the Mae Nam Chao Phraya subpopulation, may reflect a bottle neck created by a recent change of river course due to local geographic changes.

Magtoon et al. (1992) further described the evolved karyotype of *O. minutillus* as being subdivided into two phases, more primitive (0–2 LM-chromosomes, 2n=42−40) and more developed (8–14 LM-chromosomes, 2n=34−28), as expressed by the degree of chromosome rearrangements. The fact that chromosome rearrangements were found only in the Mae Nam Chao Phraya subpopulation suggests that the series of chromosome rearrangements had developed after the three subpopulations had become allopatrically isolated.

Little allozymic difference was found between the two karyotypes of the evolved type in the Mae Nam Chao Phraya subpopulation. However, no karyotype differences were detected between the Peninsular and the Mekong subpopulations, which have many allozymic differences and a relatively large genetic distance. This suggests that karyotype evolution and allozymic evolution does not always occur in parallel, and that karyotype evolution may proceed at a faster rate.

Sakaizumi et al. (1983) and Sakaizumi (1986) estimated that the genetic distance among the four populations of *O. latipes* in China, the Korean Penin-

sula and the Japanese Archipelago exceeded 0.4, but suggested that the populations were, nevertheless, conspecific. Chen et al. (1989) also regarded *O. latipes* as a single species and subdivided it into *O. l. latipes*, from Japan and eastern Korea (2n=48), and *O. l. sinensis*, from China and western Korea (2n=46). In the case of *O. minutillus*, the largest genetic distance among the 11 local populations in Thailand was 0.123. Tsuyuki et al. (1988) demonstrated successful meiosis in F_1 hybrids between different karyotypes and obtained an F_2 generation in *O. minutillus*. Considering the genetic information presented here, the occurrence of fertile F_1 progeny between *O. minutillus* of different karyotypes, and the relative magnitude of genetic difference in the related species, *O. latipes*, it is apparent that *O. minutillus* subpopulations in Thailand should be regarded as conspecific in spite of their genetic differentiation and karyotype polymorphism.

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タイメダカ *Oryzias minutillus* の遺伝的分化

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タイメダカ *Oryzias minutillus* は，タイ，ミャンマー，および，中国の雲南省に分布する．本種には核型の多型が知られており，28 本から 42 本の間で染色体数が変異する．本研究では，タイメダカの地理的分布に沿った遺伝的分化い程度と核型多型との関係を明らかにするために，タイ各地から 11 個体群を採集し，酵素多型を用いて集団遺伝学的解析を行った．

調査された 24 遺伝子座のうち，*Est*, *Pgm*, *Gpi-3*, および *Me* の 4 遺伝子座において，タイメダカは地域に特有な対立遺伝子を有していた．この 4 遺伝子座の対立遺伝子の特徴と 24 遺伝子座に基づくデンドログラムから，調査した 11 個体群は，マレー半島部の半島集団，チャオプラヤ川水系を中心にしたチャオプラヤ集団，そして，メコン川水系に属するメコン集団の 3 つの遺伝的集団から構成されていることが明らかになった．3 つの集団の分布域は大河川流域あるいは地峡部に形成されており，これらの遺伝的集団は，山脈等の地形的要因により遺伝的交流が妨げられた結果形成されたと推測される．

3 つの遺伝的集団と報告されている核型を比較すると，半島集団とメコン集団はいずれも，腕数が 42, NORs が端部染色体に位置することで特徴づけられる basic type の核型を有しており，染色体数にも変異はなかった．一方，チャオプラヤ集団は腕数が 44, NORs は次中部型染色体にあることで特徴づけられる evolved type の核型から構成され，染色体数も 42 本から 28 本の間で変異していた．このことは，3 つの遺伝的集団が分化した後に，チャオプラヤ集団内だけで核型の多型が生じたことを示唆している．

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