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A Supplementary Study on the Divergence of Japanese Fishes of the Genus *Neoclinus*

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Abstract Electrophoretic analyses were made on the two species of *Neoclinus, N. nudus* and *N. okazakii*, from Okinawa in addition to the five congeneric species (including *N. okazakii*) from Shirahama examined in the previous study (Fukao and Okazaki, 1987). In terms of Nei's genetic distance, *N. nudus* was grouped with *N. lacunicola* and *N. toshimaensis* (*N. lacunicola* species complex) with relatively small values. Recognition of *N. okazakii* from Okinawa was supported by the present electrophoretic study. The small genetic distance of 0.001 was scored between Okinawa and Shirahama populations of this species.

Electrophoretic analyses of genetic differences are useful since biochemical genetic variation is a sensitive and powerful indicator of systematic relationships of organisms (Avise, 1974), particulary for closely related or morphologically indistinguishable taxa (Bucklin, 1985).

In our previous study (Fukao and Okazaki, 1987), electrophoretic analyses were made on the five species of Japanese *Neoclinus* co-occurring in temperate Shirahama, Wakayama Prefecture. The results showed that the five species were well isolated from one another genetically, each pair of species with clearly distinctive allele in some loci, and that they could be divided into two major groups based upon Nei's genetic distances (*D*): viz., one consists of three members of *N. bryope* species complex (*N. bryope, N. chihiroe*, and *N. okazakii*) and the other of *N. lacunicola* and *N. toshimaensis*. Of the five species, *N. okazakii* also occurred in subtropical Okinawa Island where another species, *N. nudus*, co-occurred (Fukao, 1990). Then, the electrophoretic analyses for the Japanese fishes of *Neoclinus* were conducted again including *N. nudus* and *N. okazakii* from Okinawa for the purpose of elucidating the relationships among the fishes, following the background cited at the beginning.

Materials and methods

During the period from April 28 to May 2, 1985, samplings were made by using SCUBA and a dip net in the waters of Heshikiya, Okinawa Island (28°18′N and 127°56′E). Additional fishes of 5 species from Shirahama (33°41′N and 135°20′E) were also collected in the waters around the Seto Marine Biological Laboratory from June 24 to June 28, 1985. The fishes collected from Heshikiya were transported alive to the senior author's laboratory and reared about a month there. Then, they were frozen and stored at −20°C until analyzed. The additional fishes collected from Shirahama were transported alive directly to the laboratory and stored at −20°C until analyzed.

Genetic data were collected from analysis of 14 enzymes. Examined enzymes and their abbreviations (in parentheses) were as follows. Aspartate aminotransferase (*AAT*); aconitase (*ACON*); adenylate kinase (*AK*); creatine kinase (*CK*); β-galactosidase (*β-GAL*); glycyl-leucine aminopeptidase (*pep-GL*); isocitrate dehydrogenase (*IDH*); lactate dehydrogenase (*LDH*); leucylglycylglycine aminopeptidase (*pep-LGG*); malate dehydrogenase (*MDH*); 6-phosphogluconate dehydrogenase (*6-PGD*); phosphoglucomutase (*PGM*); phosphomannose isomerase (*PMI*); superoxide dismutase (*SOD*). Locus designation, tissue distribution and the used buffer systems were the same as those in the previous study (Fukao and Okazaki, 1987). The procedure of electrophoresis also followed the previous study. Alleles on gels were scored by arbitrarily designating the most common allele at each locus in *N. lacunicola* as the standard "100" allele. Other alleles were assigned numerical names based on their mobilities relative to that of the standard allele and the origin. The original data used in the previous study were included in the present study. The total number of fish

Table 1. Gene frequencies at 19 loci in Japanese species of *Neoclinus*. Nb (SH), *N. bryope* from Shirahama; Nc (SH), *N. chihiroe* from Shirahama; No (SH), *N. okazakii* from Shirahama; No (OK), *N. okazakii* from Okinawa; Nl (SH), *N. lacunicola* from Shirahama; Nt (SH), *N. toshimaensis* from Shirahama; Nn (OK), *N. nudus* from Okinawa. The numbers of fish examined are given in parentheses.

Locus	Allele	Nb (SH) (72)	Nc (SH) (46)	No (SH) (28)	No (OK) (24)	Nl (SH) (90)	Nt (SH) (88)	Nn (OK) (10)
AAT-1		1.000	1.000	1.000	1.000	1.000	1.000	1.000
AAT-2	65	0.012	0.043					
	80	0.976	0.935					
	100	0.012	0.022	0.769	0.929	1.000	1.000	1.000
	110			0.231	0.071			
ACON	80	1.000					1.000	1.000
	100		1.000	1.000	1.000	1.000		
AK		1.000	1.000	1.000	1.000	1.000	1.000	1.000
CK	90		1.000					
	100	1.000				1.000	1.000	1.000
	110			1.000	1.000			
β-GAL	90							0.100
	100	1.000	1.000	1.000	1.000	1.000	1.000	0.900
Pep-GL	70						0.284	
	80						0.710	
	90	0.457	0.022			0.322	0.006	
	100	0.543	0.978	0.857	0.958	0.678		1.000
	110			0.143	0.042			
IDH-1	70	0.008						
	85	0.992					0.994	1.000
	100		1.000	1.000	1.000	1.000	0.006	
IDH-2	70					0.006	0.006	
	100	1.000	1.000	1.000	1.000	0.983	0.994	1.000
	130					0.011		
LDH-1		1.000	1.000	1.000	1.000	1.000	1.000	1.000
LDH-2	70	1.000	0.074				0.006	
	100					1.000	0.994	1.000
	115		0.926	1.000	1.000			
LDH-3		1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pep-LGG	85	1.000						
	100		1.000	1.000	1.000	1.000	1.000	1.000
MDH-1	70	1.000	1.000	0.957	1.000			
	100					0.978	0.994	1.000
	120					0.017	0.006	
	210			0.043				
	270					0.006		
MDH-2	70	0.008			0.042			
	100					0.972	1.000	1.000
	105	0.992	1.000	1.000	0.958			
	110					0.028		
6-PGD	80	0.008						
	95					0.006		
	100	0.992			0.021	0.978	1.000	1.000
	110					0.017		
	120		1.000	1.000	0.979			
PGM	40	0.025						
	50	0.008						
	70	0.967	0.029			0.028		
	80		0.015			0.006		
	100		0.956	0.978	0.917	0.967	1.000	1.000
	120			0.022	0.083			

(Table 1, continued)

Locus	Allele	Nb (SH) (72)	Nc (SH) (46)	No (SH) (28)	No (OK) (24)	Nl (SH) (90)	Nt (SH) (88)	Nn (OK) (10)
PMI	70		0.015					
	90			0.045		0.009		
	100	1.000	0.985	0.955	1.000	0.991	1.000	1.000
SOD	30						0.585	
	80		0.026					
	100					1.000	0.415	1.000
	160	0.991	0.816	1.000	0.979			
	240	0.009	0.158		0.021			

analyzed (the number of additional fish in parentheses) were 72 (31), 46 (19), 28 (16), 24, 90 (26), 88 (48), and 10 for *N. bryope* from Shirahama, *N. chihiroe* from Shirahama, *N. okazakii* from Shirahama, *N. okazakii* from Okinawa, *N. lacunicola* from Shirahama, *N. toshimaensis* from Shirahama, and *N. nudus* from Okinawa, respectively. Calculations of average heterozygosity and of genetic distance followed Nei (1978).

Results and discussion

The same 19 loci in 14 enzyme systems as those in the previous study (Fukao and Okazaki, 1987) were surveyed in all of the six Japanese species of *Neoclinus* including 2 local populations of *N. okazakii*. Genetic variants were observed in 15 of these 19 loci, while no variant was scored in the remaining 4 loci. Gene frequencies are shown in Table 1. The present analysis is supplementary to the previous study. The main point is that *N. nudus* from Okinawa and Okinawa population of *N. okazakii* are newly included in the analysis for Japanese *Neoclinus*.

In *N. nudus*, genetic variants were detected only in a single locus of β-galactosidase (the dominant *β-Gal*-100 and the lesser *β-Gal*-90). In this locus, all the other 5 species were fixed to the allele *β-Gal*-100.

Fukao (1987) noted that the fish from Okinawa reported by Stephens (1961) may be *N. okazakii, N. chihiroe*, or the fourth form of *N. bryope* species complex. All the examined fish of *N. bryope* complex from Okinawa showed a two-banded electromorph at *CK* locus which was regarded as monomorphic for *Ck*-110 and as the most striking characteristic of *N. okazakii* (Fukao and Okazaki, 1987). A morphological examination including the two specimens reported by Stephens (1961) also clearly showed that the fish from Okinawa was referable to *N. okazakii* (Fukao, 1990). Okinawa population of *N. okazakii* possessed the following three rare variants not detected in Shirahama population: viz., *Mdh-2*-70, *6-Pgd*-100, and *Sod*-240. Conversely, the two alleles, *Mdh-1*-100 and *Pmi*-90, were not detected in Okinawa population but rarely in Shirahama population. In either case, the population without the above rare variants was fixed to the respective dominant allele in the counterpart population. In these two populations of *N. okazakii*, the common genetic variants were observed in *AAT-2, Pep-GL*, and *PGM* loci, each with rather slight difference in gene frequency. Sufficient samples from various localities are needed for interpretation of the above noted difference in gene composition between populations. Among the common variants, the allele, *Pgm*-120, was not detected in the previous study at all but was detected in both the populations in the present study. Among others, the following variants were newly detected in the present study: viz., *Idh-1*-70 and *Mdh-2*-70 for *N. bryope*; *Pgm*-80 and *Pmi*-70 for *N. chihiroe*; *Pgm*-80 for *N. lacunicola*; *Pep-Gl*-90 and *Idh-1*-100 for *N. toshimaensis*. All these newly detected variants were rare and observed in only one individual of the species except for *Pgm*-120 for Okinawa population of *N. okazakii* (observed in 2 individuals). As for the 5 species from Shirahama, additional fishes were collected in late June, 1985. The original data in the previous study came from fishes collected in early May and middle July, 1984. Although the above noted rare alleles were newly detected, no significant difference between fishes collected in 1984 and those in 1985 was observed in gene frequencies of any locus. Both the samples contained newly settled young and adults of seemingly 1 year old or more in each species. These fishes are sedentary and believed not to migrate after settling from pelagic larval life. Judging from these, no significant effect on the re-

presentation of each population (i.e., allelic composition) was just expected from the present time lag in sampling noted above. Then, only the combined data were represented and the subsequent analyses were made based on the combined data.

Summary of genetic variability is shown in Table 2. Proportion of polymorphic loci of six Japanese species of *Neoclinus* including 2 local populations of *N. okazakii* ranged from 0.053 to 0.316 or from 0.053 to 0.158 when a locus was considered polymorphic in populations in which the frequency of the most common allele was less than 0.95 (Criterion 1) or than 0.99 (Criterion 2), respectively. The range of observed and expected average heterozygosities of species or populations were 1.1 to 4.8% and 1.0 to 5.0%, respectively. The obtained values between observed and expected heterozygosities (expected on the basis of Hardy-Weinberg assumptions) in each species or population were almost identical. The trend among species or populations observed in proportion of polymorphic loci was not necessarily consistent with that in average heterozygosity in the present study. The proportion of polymorphic loci is considered to be a relatively poor index to degree of genetic variation in populations because it is strongly dependent upon sample size (Selander et al., 1971). The average heterozygosities observed in 5 species from Shirahama were near the mean for fish ($\bar{H}$= 0.054 for 77 species by Avise and Aquadro, 1982; $\bar{H}$=0.051 for 51 species by Nevo, 1978; $\bar{H}$=0.055 for 106 species by Smith and Fujio, 1982). On the other hand, the value observed in *N. nudus* from Okinawa was considerably less than those of 5 species from Shirahama, though the number of fish examined was relatively small in this species. In this species, small population size was expected from their extremely narrow habitat (Fukao, 1990). Although the present result may be inconclusive because of the limited number of fish examined, when coupled with the above expectation of small population size, the effect of genetic drift is conceivable in the population. The values obtained in Okinawa population of *N. okazakii* were also somewhat lower than those in Shirahama population. In subtropical Okinawa, this species was also believed to be restricted to the particular habitat exceptionally unoccupied by flourishing blenniids, though the habitat was seemingly not so extremely narrow as compared to that of *N. nudus*. In temperate Shirahama, this species shows relatively less constrained habitat

Table 2. Proportion of polymorphic loci and average heterozygosity for examined loci in Japanese fishes of *Neoclinus*. SH, Shirahama; OK, Okinawa. Criterion 1: The frequency of the most common allele is ≤.95. Criterion 2: The frequency of the most common allele is ≤.99.

Species (Loc.)	Polymorphic loci		Average heterozygosity	
	Criterion 1	Criterion 2	Observed	Expected
N. nudus (OK)	0.053	0.053	0.011	0.010
N toshimaensis (SH)	0.105	0.105	0.048	0.050
N. lacunicola (SH)	0.053	0.316	0.037	0.037
N. bryope (SH)	0.053	0.158	0.038	0.036
N. chihiroe (SH)	0.158	0.316	0.034	0.039
N. okazakii (SH)	0.105	0.263	0.043	0.044
N. okazakii (OK)	0.105	0.316	0.029	0.029

Table 3. Nei's (1978) measure of genetic distance (above diagonal) and fractions of diagnostic loci (below diagonal) in comparison among 6 Japanese species of *Neoclinus* (including 2 local populations of *N. okazakii*). SH, Shirahama; OK, Okinawa.

Species (Loc.)	Nn (OK)	Nt (SH)	Nl (SH)	Nb (SH)	Nc (SH)	No (SH)	No (OK)
N. nudus (OK)		0.064	0.121	0.484	0.633	0.570	0.552
N. toshimaensis (SH)	1/19		0.174	0.503	0.706	0.624	0.615
N. lacunicola (SH)	2/19	3/19		0.656	0.457	0.400	0.389
N. bryope (SH)	7/19	8/12	9/19		0.478	0.565	0.563
N. chihiroe (SH)	9/19	10/19	7/19	7/19		0.103	0.108
N. okazakii (SH)	8/19	9/19	6/19	8/19	2/19		0.001
N. okazakii (OK)	8/19	9/19	6/19	8/19	2/19	0/19	

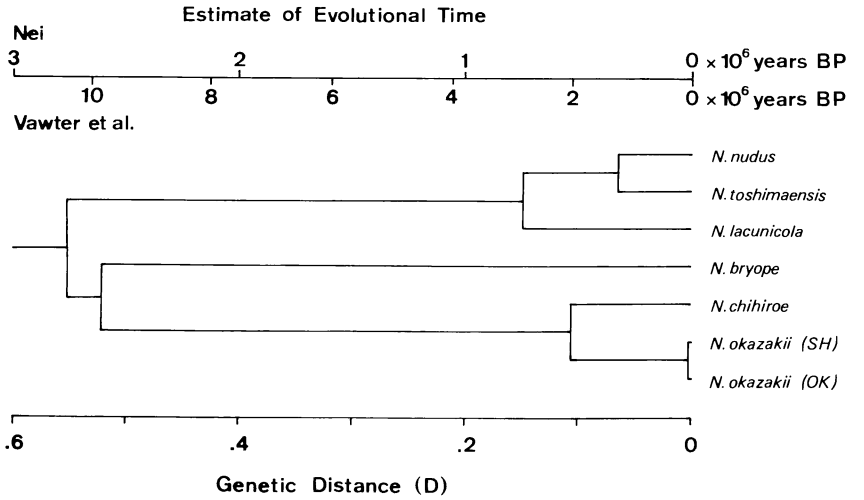

Fig. 1. Phenetic dendrogram produced using UPGMA procedure of cluster analysis on Nei's *D* values given in Table 3, with two different estimates of evolutional time (Nei, 1975; Vawter et al., 1980). SH, Shirahama population; OK, Okinawa population.

preference (Fukao and Okazaki, 1987). Aside from the effect of genetic drift, it is possible that intrapopulation genetic variability is directly related to some aspect of ecological amplitude or width of ecological niche exploited by the population, which in turn varies geographically (Selander et al., 1971). Smith and Fujio (1982) reported that low variability was observed rather in habitat generalists than in habitat specialists in marine teleosts, leaving the studies on intraspecific populations or closely related conspecifics as the profitable ones for future research. In the treatise, they also assumed that the specialist species or population represents an accumulation of several narrow-range alleles coming from individuals adapted to microhabitats and is characterized by high heterozygosities. The present case did not conflict with their assumption because the low variability in Okinawa population was assumed to come from the reduced variability in microhabitat.

Table 3 shows matrices of values of Nei's (1972) genetic distance (*D*) and the fraction of diagnostic loci (Ayala and Powell, 1972) in gene-enzyme. Fig. 1 represents a phylogenetic tree constructed from indices of genetic distance according to the unweighted paired-group method (UPGMA: Sneath and Sokal, 1973). Crude estimates of divergence time presented by Nei (1975) and Vawter et al. (1980) are also shown in Fig. 1.

No significant difference from the previous study was observed in the relationship among the 5 species from Shirahama. In cases of true sympatry (in space and time), genetically differentiated species are easily recognized when fixed allelic differences are detected (Shaklee et al., 1982). In the comparison among the 5 species, fixed allelic difference was detected in 1 to 8 loci (2 to 10 diagnostic loci were present). Thus, the 5 sympatric forms in Shirahama were proved again to be discrete biological species.

The genetic distance of 0.001 was scored between Okinawa and Shirahama populations of *N. okazakii*. This value is lower than the lower limit of the range at population level (0.002–0.065; $\bar{x}=0.05$) proposed by Shaklee et al. (1982). However, some minor morphological differences (in total number of free tips of nasal cirri, number of lateral line pores, and proportions of some body parts) were observed between these two populations (Fukao, 1990). As assumed among the Japanese local populations of the anemonefish *Amphiprion clarkii* (Bell et al., 1982), relative isolation of population may be maintained by mostly localized larval dispersal with some probability of genetic exchange by the larval transport on the Kuroshio.

From the UPGMA of genetic distance, *N. nudus* was grouped with *N. toshimaensis* and *N. lacunicola* (*N. lacunicola* species complex). The genetic distance values obtained between *N. nudus* and the other two species (0.064 and 0.121) were relatively low among values obtained in congeneric fish species pair (Shaklee et al., 1982; Wallis and Beadmore,

1984). Morphologically, however, similarity between *N. lacunicola* and *N. toshimaensis*, proved to be discrete biological species, was seemingly superior to that between *N. nudus* and them (Fukao, 1990). The distinct morphological gap including an osteological feature, presence or absence of a minimal hypural, between *N. nudus* and the other species makes little doubt that they should be treated as distinct species. Fixed allelic difference was also detected in *Pep-GL* locus in the case between *N. nudus* and *N. toshimaensis* and in *ACON* and *IDH-1* loci in the case between *N. nudus* and *N. lacunicola*, though it was not definitely effective for recognition of genetically differentiated species in the above cases of allopatry. The present results seem to suggest that these three species might be derived from a common ancestral stock rather recently.

As improvement on our past discussion about speciation in the Japanese species of *Neoclinus* will be published elsewhere.

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日本産コケギンポ属魚類の分化について—補足—

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Fukao and Okazaki (1987) で報告した和歌山県白浜産5種に沖縄産のアライソコケギンポ *Neoclinus okazakii*（白浜にも出現）およびハダカコケギンポ *N. nudus*（白浜に出現せず）を加えて，日本産コケギンポ属 *Neoclinus* 6種の遺伝的分化及び異同について，アイソザイムにより検討した．Nei (1978) の遺伝的距離からハダカコケギンポは白浜産のイワアナコケギンポ *N. lacunicola* とトーシマコケギンポ *N. toshimaensis* に極めて近い類縁関係を

示すことが判明した (*N. lacunicola* species complex). 別の1群 (*N. bryope* species complex) に属するアライソコケギンポは，沖縄産の個体も全てアイソザイム分析から同種であることが支持された．なお，この種の沖縄産と白浜産の間の遺伝的距離は0.001であった．

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