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

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Abstract Five Japanese species of *Neoclinus* from Shirahama, Japan were studied based on allelic frequencies at 19 genetic loci. Habitat partitioning and overlap in these five species in the waters of Shirahama was also observed. The five species which cooccur in Shirahama are well isolated genetically, each pair of species with clearly distinctive allele in some loci. A resulting phylogenetic tree among five species of Japanese *Neoclinus* based upon Nei's genetic distances (*D*) indicates that the Japanese *Neoclinus* could be divided into two major groups. One consists of *N. lacunicola* and *N. toshimaensis* and another three species of *N. bryope* complex (i.e. *bryope*, *chihiroe* and *okazakii*). This agrees well with the grouping based on morphology. Three members of *N. bryope* complex showed an imperfect habitat partitioning. However, the obtained results clearly indicate that gene exchanges have not occurred among these three members. *N. bryope* mainly inhabits tide pools (TP habitat), *N. chihiroe* mainly in the upper subtidal of moderately exposed rocky reefs (ME habitat), and *N. okazakii* mainly in the upper subtidal of very exposed rocky reefs (VE habitat). The two species of another major group showed more rigid habitat partitioning. *N. lacunicola* mainly inhabits ME habitat where it predominates over *N. chihiroe* and *N. toshimaensis* inhabits VE habitat where it predominates over *N. okazakii*.

The genus *Neoclinus* occupies a rather isolated position in the family Clinidae and is believed to be closest to the clinid stock that gave rise to the fishes of the family Chaenopsidae (Stephens, 1963). Springer (1955) tentatively estimated that *Neoclinus* was derived from ancestors of the tribe Paraclinidi of the clinid subfamily Labrisominae. Later, George and Springer (1980) elevated the subfamily Labrisominae to family rank. Lindquist (1981) presumed that *Neoclinus* belongs to the family Labrisomidae. On the other hand, Lindberg and Krasyukova (1975), the Ichthyological Society of Japan (1981) and Nelson (1984) included *Neoclinus* in the family Chaenopsidae.

Nine species were recognized in the genus *Neoclinus*. Three species, *N. blanchardi* Girard, *N. uninotatus* Hubbs and *N. stephensae* Hubbs are known only from California (Hubbs, 1953; Stephens, 1961; Stephens and Springer, 1971). Five species of *N. bryope* (Jordan et Snyder), *N. chihiroe* Fukao, *N. okazakii* Fukao, *N. lacunicola* Fukao and *N. toshimaensis* Fukao are known only from Japan (Fukao, 1980, 1987), and *N. nudus* Stephens et Springer is known only from Taiwan (Stephens and Springer, 1971). Stephens and Springer (1971) noted that a specimen collected from Korean waters was regrettably lost. The fish could possibly be *N. lacunicola* or *N. toshimaensis* (Springer, pers. comm.). Thus, the genus *Neoclinus* shows an interesting distribution pattern of amphi-Pacific. The restriction of the eastern Pacific species to the temperate California in the New World has been regarded as the result in which the fish were replaced by the more specialized group, Chaenopsidae, with similar habitat preference in the tropics (Stephens, 1961; Stephens and Springer, 1971). The Japanese forms have been believed to be emigrated from the New World to Japan through the northern Pacific (Hubbs, 1952, 1953; Stephens, 1961; Fukao, 1980).

The senior author revised the Japanese species by referring to their habitat preference (Fukao, 1980). Later, it was proved that *N. bryope* in the revision contains three species of *N. bryope*, *N. chihiroe* and *N. okazakii* (*N. bryope* species complex) (Fukao, 1987), based on the electrophoretic analysis described in detail in the present study. The present study was conducted for further elucidation on the divergence of Japanese forms.

Materials and methods

Field observations and samplings. During the years from 1973 to 1977 and in May and July of 1984, underwater observations and samplings were made by using SCUBA around the Seto Marine

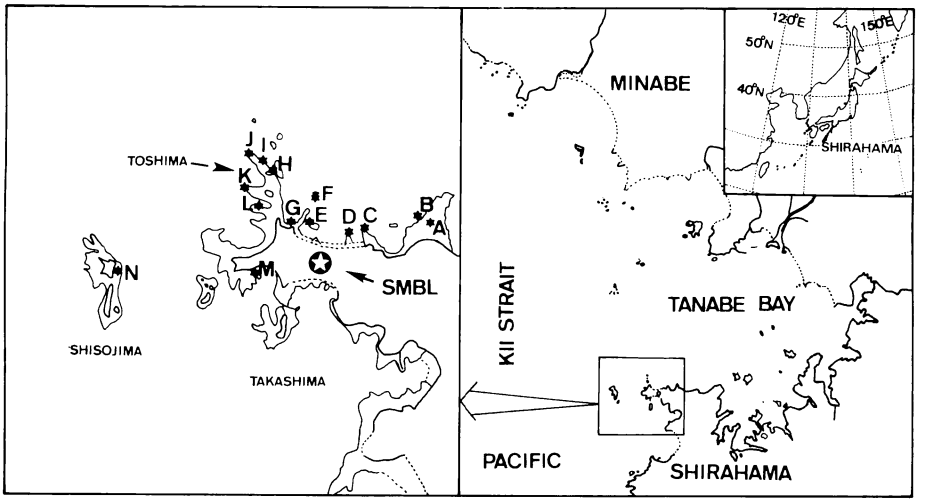


Fig. 1. Study area and sampling stations. SMBL, Seto Marine Biological Laboratory.

Biological Laboratory which is located at the mouth of Tanabe Bay on the west coast of the Kii Peninsula (approximately 33°41′N and 135° 20′E; Fig. 1). At low tide, observations and samplings were sometimes extended to tide pools. Samplings were made using a dip net and anesthetic quinaldine. Habitat category, range of depths and major topographical features of each station were presented in Table 1. In habitat categories, the very exposed rocky reefs (VE) were characterized by the settlement of a barnacle, *Balanus tintinnabulum volcano*, while the moderately exposed rocky reefs (ME) were the exposed reefs without the barnacles. In addition to these, the stations where barnacles were rarely found were expressed as ME-VE. The last category was tide pools (TP) in the intertidal of exposed reefs.

Table 1. Habitat category, range of water depths (under the low water level) and topography of each station. TP, tide pools (intertidal rocky reefs); ME, upper subtidal of moderately exposed rocky reefs; VE, upper subtidal of very exposed rocky reefs; ME-VE, upper subtidal of rocky reefs intermediate between ME and VE in the degree of exposure (see text).

Station	Habitat	Water depth (m)	Topography
A	TP	—	
B	ME-VE	0–4	slope
C	ME-VE	0–3	slope, flat, cliff
D	ME-VE	0–4	slope, flat, cliff
E	ME	0–5	slope, flat, boulders
F	ME	4–8	slope, flat
G	TP	—	
H	VE	0–4	slope
I	VE	0–5	cliff
J	VE	0–4	slope, flat
K	VE	0–3	slope, flat, cliff
L	VE	0–3	cliff
M	TP	—	
N	VE	0–3	slope, flat

Electrophoresis. The total numbers of fishes analyzed were 41, 27, 12, 64 and 40 for *N. bryope*, *N. chihiroe*, *N. okazakii*, *N. lacunicola* and *N. toshimaensis*, respectively. Most of the fishes were collected from the waters around the Seto Marine Biological Laboratory in early May and middle July, 1984. Sampling stations were A, E, G, J, K and M in Fig. 1. Four individuals of *N. bryope* collected by Mr. Tanase from tide pools in the shore of the town Minabe, Wakayama Prefecture (Fig. 1) in early June, 1984 were reared about a month in the Kyoto University Aquarium adjoining to the Laboratory. These fishes were frozen and shipped to the Far Seas Fisheries Research Laboratory, Shimizu, Shizuoka Prefecture, and stored at −20°C until analyzed.

Liver, skeletal muscle and eyeball were taken from each specimen and used for analysis. Processing of samples and electrophoretic methods have been described elsewhere (May et al., 1979; Okazaki, 1982). The following five buffer sys-

tems were used: 1) an amine (N-(3-Aminopropyl)-morpholine) citrate buffer (pH 6.5) described by Clayton and Tretiak (1972; abbreviated as 'AC'); 2) an amine (N-(3-Aminopropyl)-diethanolamine) citrate buffer (pH 7.0) described by Numachi et al. (1979) with slight modification based on the Clayton and Tretiak (1972; abbreviated as 'AEA'); 3) a citric-Tris buffer (pH 7.0) described by Siciliano and Shaw (1976; abbreviated as 'CT'); 4) a Tris-boric acid-EDTA buffer (pH 8.5) described by Markert and Faulhaber (1965; abbreviated as 'MF'); 5) a discontinuous Tris-citric acid (gel pH 8.5), lithium hydroxideboric acid (tray pH 8.5) buffer systems described by Ridgway et al., (1970; abbreviated as 'RW'). Staining procedures followed the method of Harris and Hopkinson (1976).

Genetic data were collected from analysis of 14 enzymes (Table 2). The method for allele designation were adapted from that proposed by Allendorf and Utter (1979). 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. Nei's (1972) genetic distance (*D*) measure was used to quantify the degree of similarity between populations and dendrograms were derived using UPGMA (Sneath and Sokal, 1973).

Results

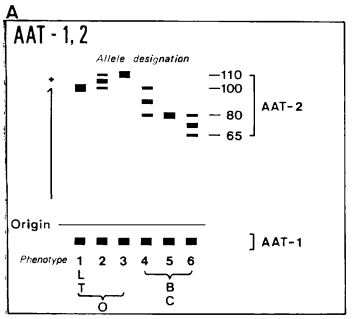
Genetic features of five Japanese *Neoclinus*. In the absence of the breeding data, the Mendelian nature of the electrophoretic variants was inferred from the banding patterns on the gels under the following criteria: 1) banding patterns had to be consistent with the known molecular structure of that protein; 2) when a gene is expressed in more than one tissue, variant phenotypes should be parallel among tissues (Grant et al., 1983).

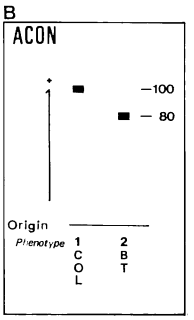
Nineteen genetic loci were surveyed in 14 enzyme systems in all of the five species of *Neoclinus*. Genetic variants were observed in 14 of these 19 loci, while no variant was scored in the remaining 5 loci. Gene frequencies were shown in Table 3. In the followings, genetic interpretation of banding patterns were described.

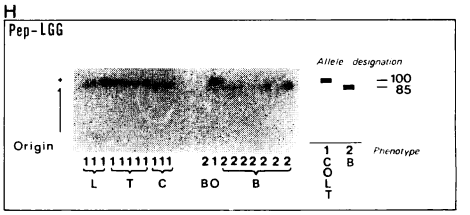
Aspartate aminotransferase (*AAT-1, 2*; Fig. 2A): Two loci were resolved for the aspartate aminotransferase. The cathodal locus, *AAT-1*, was monomorphic for all five species and no difference was noticed in the mobility among these species. Four alleles, designated as *Aat-2*-65, -80, -100 and -110 were present at the anodal locus, *AAT-2*. *N. lacunicola* and *N. toshimaensis*

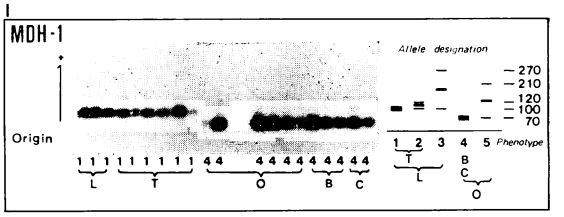
Table 2. Enzymes, abbreviations, locus designation, tissue distribution and buffer systems (see text for the designation of the buffer systems). * E, eye; L, liver; M, muscle.

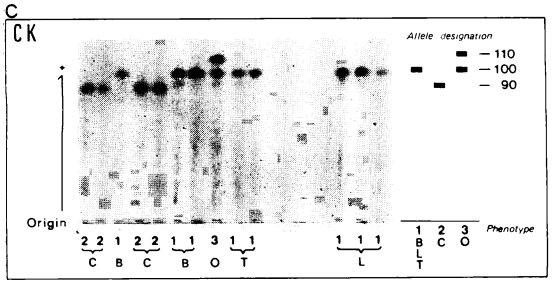
Enzyme	Abbreviation	Locus designation (if multiple)	Tissue* distribution	Buffer system
Aspartate aminotransferase	*AAT*	1	M	AC
		2	E	RW
Aconitase	*ACON*		M	CT
Adenylate kinase	*AK*		M	AC
Creatine kinase	*CK*		M	RW
β-Galactosidase	*β-GAL*		L	RW
Glycyl-leucine aminopeptidase	*Pep-GL*		E, M	RW
Isocitrate dehydrogenase	*IDH*	1	M	AEA
		2	L	AEA
Lactate dehydrogenase	*LDH*	1, 2	L, M	RW
		3	E	RW
Leucylglycylglycine aminopeptidase	*Pep-LGG*		E, M	RW
Malate dehydrogenase	*MDH*	1	L	AC
		2	M	AC
6-Phosphogluconate dehydrogenase	*6-PGD*		M	AEA
Phosphoglucomutase	*PGM*		M	AEA
Phosphomannose isomerase	*PMI*		E	AC
Superoxide dismutase	*SOD*		L	MF

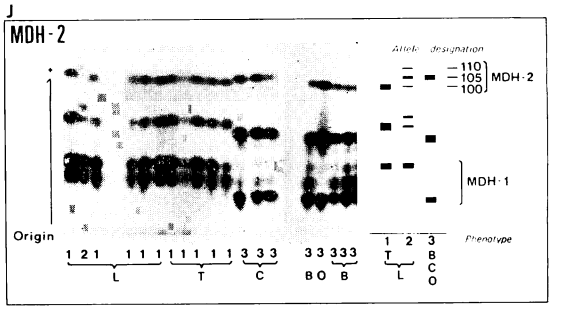

A
AAT-1,2
Allele designation
−110
−100
−80
−65
AAT-2
Origin
AAT-1
Phenotype 1 2 3 4 5 6
L T O B C

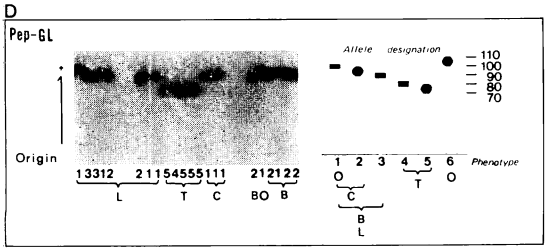

B
ACON
−100
−80
Origin
Phenotype 1 2
C B
O L T

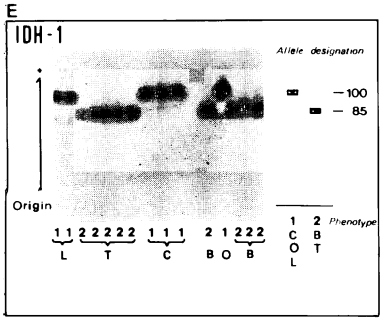

H
Pep-LGG
Allele designation
−100
−85
Origin
111111111111 212222222
L T C BO B
1 2 Phenotype
C B
O L
T

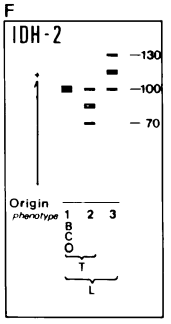

I
MDH-1
Allele designation
−270
−210
−120
−100
−70
Origin
1 1 1 1 1 1 1 1 44 44 4 4 4 4 4 4 4
L T O B C
1 2 3 4 5 Phenotype
T B
L C
O

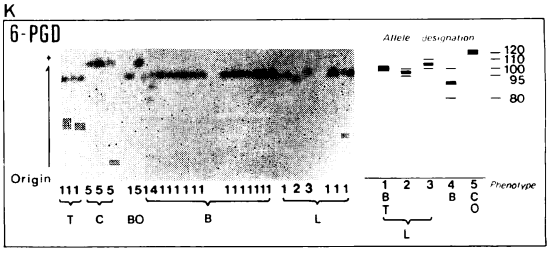

C
CK
Allele designation
−110
−100
−90
Origin
2 2 1 2 2 1 1 3 1 1 1 1 1
C B C B O T L
1 2 3 Phenotype
B C
L O T

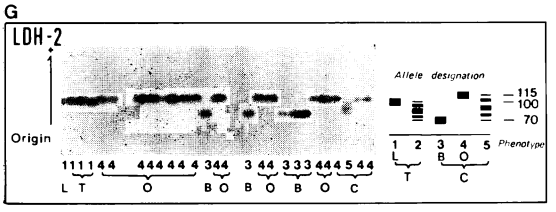

J
MDH-2
Allele designation
−110
−105
−100
MDH-2
MDH-1
Origin
1 2 1 1 1 1 1 1 1 1 3 3 3 3 3 3 3 3
L T C BO B
1 2 3 Phenotype
T B
L C
O

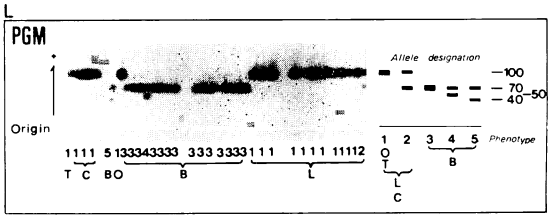

D
Pep-GL
Allele designation
−110
−100
−90
−80
−70
Origin
13312 2115455511 1 2121 22
L T C BO B
1 2 3 4 5 6 Phenotype
O T O
C
B
L


E
IDH-1
Allele designation
−100
−85
Origin
1 1 2 2 2 2 2 1 1 1 2 1 2 2 2
L T C BO B
1 2 Phenotype
C B
O T
L


F
IDH-2
−130
−100
−70
Origin
phenotype 1 2 3
B
C
O
T
L


K
6-PGD
Allele designation
−120
−110
−100
−95
−80
Origin
111 5 5 5 15141111111 1111111 1 2 3 111
T C BO B L
1 2 3 4 5 Phenotype
B B C
T O
L


G
LDH-2
Allele designation
−115
−100
−70
Origin
1111 4 4 444 4 4 4 4 344 3 44 3 3 3 444 5 44
L T O B O B O B O C
1 2 3 4 5 Phenotype
L B O
T C


L
PGM
Allele designation
−100
−70
−40
−50
Origin
1111 5 133343333 333 3331 1 1 1111 11112
T C BO B L
1 2 3 4 5 Phenotype
O B
T
L
C

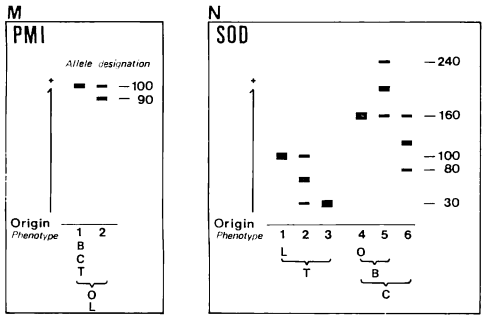


Fig. 2. Starch gel patterns of enzyme variants in five Japanese species of *Neoclinus*. At each locus, each variant phenotype was designated by a number. B, *N. bryope*; C, *N. chihiroe*; L, *N. lacunicola*; O, *N. okazakii*; T, *N. toshimaensis*.

were fixed for allele *Aat-2*-100. *N. bryope*, *N. chihiroe* and *N. okazakii* were polymorphic at this locus and these three species shared allele *Aat-2*-100 in common. The gene frequencies for *Aat-2*-100 in *N. bryope* and *N. chihiroe* were low, while those in *N. okazakii* were predominant. An allele *Aat-2*-110 was exclusively observed in *N. okazakii*. Besides the rare *Aat-2*-100, *N. bryope* and *N. chihiroe* shared the predominant *Aat-2*-80 and another rare *Aat-2*-65.

Aconitase (*ACON*; Fig. 2B): Two loci were presumably present in this enzyme system, but the slow locus could not be reliably interpreted because of the diffusion of the bands. The fast locus was monomorphic for all 5 species. *N. lacunicola*, *N. chihiroe* and *N. okazakii* were fixed for allele *Acon*-100, while *N. toshimaensis* and *N. bryope* fixed for *Acon*-80.

Adenylate kinase (*AK*): *AK* was monomorphic for all 5 species and the mobility was identical for all species.

Creatine kinase (*CK*; Fig. 2C): Creatine kinase from muscle is demonstrated to be dimers for a broad taxonomic range of fishes (Ferris and Whitt, 1978) and explained to be encoded by two loci, *CK-1* and *CK-2*, in the case of salmonids (Utter et al., 1979). In the present study, all individuals of *N. lacunicola*, *N. toshimaensis*, *N. bryope* and *N. chihiroe* showed single banded electromorphs. The former three species fixed for allele *Ck*-100 and the latter for *Ck*-90. On the other hand, all individuals of *N. okazakii* showed two-banded electromorph for "alleles" *Ck*-100 and -110. Judging from the alleles possessed by *N. bryope*, *N. chihiroe* and by *N. okazakii*, the two-banded pattern of *N. okazakii* could not be regarded as hybrid as observed in the case of *CK* for the hybrid between closely related gobies, *Pomatoschistus minutus* and *P. lozanoi* (Wallis and Beardmore, 1980). Since, without exception, all individuals of *N. okazakii* showed two-banded pattern, the fact is difficult to be explained by the assumption of the absence of heteropolymers in heterozygotes for a single locus as postulated for *CK* locus of many species of fishes by Ferris and Whitt (1978). In salmonids, Utter et al. (1979) postulated that each allele is represented electrophoretically by two bands, presumably a reflection of stable posttranslational modification of a single polypeptide unit. They explained that the two-banded types are individuals with genotypes where each of four genic doses give rise to an electrophoretically identical polypeptide. From here, two possible cases could be assumed based on the present results. One is that creatine kinase from muscle is encoded by two loci, *CK-1* and *CK-2*, and both loci produce proteins of identical electrophoretic mobility for examined species except for *N. okazakii* which possessed two loci producing proteins with different mobility. Another is that the enzyme is encoded by a single locus and only *N. okazakii* possessed an allele represented electrophoretically by two bands as postulated in salmonids by Utter et al. (1979). We could not decide which case should be adopted, since no variation was observed within each species in this enzyme system. In any case, this enzyme system shows the clear distinction among three species of *N. bryope* complex. Arbitrarily, we adopted the leatter case, that this enzyme is encoded by one locus, for the calculation in the following analyses. In this context, *N. okazakii* is regarded as monomorphic for *Ck*-110.

β-Galactosidase (*β-GAL*): Two loci were present at the β-Galactosidase, but only the fast locus could be reliably scored. The slow locus could not be resolved because of the diffusion of the bands. The fast locus was monomorphic for all species and the mobility was identical for all species.

Glycyl-leucine aminopeptidase (*Pep-GL*; Fig. 2D): All five species showed the two allele polymorphism. The allele *Pep-Gl*-100 is common for *N. bryope* complex and *N. lacunicola*. Besides

this allele, *N. bryope*, *N. chihiroe* and *N. lacunicola* shared *Pep-Gl*-90, while *N. okazakii* possessed the unique allele *Pep-Gl*-110. Two alleles *Pep-Gl*-70 and -80 were unique to *N. toshimaensis*.

Isocitrate dehydrogenase (*IDH-1*, *2*; Fig. 2E, F): Two loci were scored for *IDH*. A locus, *IDH-1*, was expressed in muscle tissue and monomorphic for all species. *N. lacunicola*, *N. chihiroe* and *N. okazakii* were fixed for allele *Idh-1*-100, while *N. toshimaensis* and *N. bryope* were fixed for *Idh-1*-85. Another locus, *IDH-2*, was expressed in liver for all species. Three alleles, *Idh-2*-70, -100 and -130 were present in *N. lacunicola*. Two alleles, *Idh-2*-70 and -100 were present in *N. toshimaensis*. In both species, *Idh-2*-100 was predominant. *N. bryope*, *N. chihiroe* and *N. okazakii* were monomorphic for *Idh-2*-100.

Lactate dehydrogenase (*LDH-1*, *LDH-2* (Fig. 2G), *LDH-3*): The five electrophoretically distinguishable banding patterns for this enzyme of most mammals are well known to be tetramer formed by random association of two different subunits, each under the control of distinct genetic loci A and B (Markert, 1963). *LDH-A* predominates in skeletal muscle, while *LDH-B* in heart muscle. Many fishes have also a third locus, *LDH-E*, predominating in eyeball (Horowitz and Whitt, 1972) or in liver (De Achaval, 1984).

Three loci were present for all five species of Japanese *Neoclinus*. The slow locus, *LDH-1*, was expressed in liver tissue. All species were monomorphic with the same mobility for this locus. *LDH-2* was expressed in muscle tissue. In this locus, *N. lacunicola*, *N. bryope* and *N. okazakii* were monomorphic for allele *Ldh-2*-100, allele *Ldh-2*-70 and allele *Ldh-2*-115, respectively. On the other hand, *N. toshimaensis* possessed two alleles, *Ldh-2*-70 and -100. Two alleles, *Ldh-2*-70 and -115 were present in *N. chihiroe*. *LDH-3* presented in eye tissue was monomorphic for the same allele in all species. According to the tissue distribution of each locus, *LDH-1*, *-2* and *-3* probably correspond to the *LDH-A*, *-B* and *-E*, respectively.

Leucylglycylglyine aminopeptidase (*Pep-LGG*; Fig. 2H): A single locus, *Pep-LGG*, was monomorphic for all species. *N. bryope* was fixed for allele *Pep-Lgg*-85, while all other species were fixed for *Pep-Lgg*-100.

Malate dehydrogenase (*MDH-1*, *2*; Fig. 2I, J): It is known that malate dehydrogenase contains mitochondrial and supernatant forms. Supernatant *MDH*, examined in this study, shows a dimeric structure in many fish species (Numachi, 1970) and contains two systems under the control of separate genetic loci A and B (Bailey and Wilson, 1970). *MDH-A* and *-B* are predominant in liver and skeletal muscle, respectively (Bailey and Wilson, 1970). Two loci were also apparent in muscle tissue in the present study. The *MDH-1* locus was most clearly scored in liver tissue, suggesting this locus corresponds to the *MDH-A*. Two alleles, *Mdh-1*-100 and -120 were common between *N. lacunicola* and *N. toshimaensis*. The allele *Mdh-1*-100 was predominant in both species. Another extremely rare *Mdh-1*-270 was present in *N. lacunicola*. *N. bryope* and *N. chihiroe* were fixed for *Mdh-1*-70, while *N. okazakii* possessed the predominant *Mdh-1*-70 and the lesser *Mdh-1*-210. Another anodal locus, *MDH-2*, was expressed in muscle tissue. *N. toshimaensis* was fixed for *Mdh-2*-100. *N. lacunicola* had the predominant *Mdh-2*-100 and the lesser *Mdh-2*-110. *N. bryope*, *N. chihiroe* and *N. okazakii* were fixed for *Mdh-2*-105.

6-phosphogluconate dehydrogenase (*6-PGD*; Fig. 2K): Five alleles were resolved at this single anodal locus system. *N. toshimaensis* was fixed for allele *6-Pgd*-100. Three alleles, the predominant *6-Pgd*-100, the rare *6-Pgd*-110 and the extremely rare *6-Pgd*-95, were scored for *N. lacunicola*. The predominant *6-Pgd*-100 and the rare *6-Pgd*-80 were present in *N. bryope*. *N. chihiroe* and *N. okazakii* were fixed for *6-Pgd*-120.

Phosphoglucomutase (Fig. 2L: *PGM*): Four alleles were present at this single locus system. *N. toshimaensis* and *N. okazakii* were monomorphic for allele *Pgm*-100. *N. lacunicola* and *N. chihiroe* also shared the predominant *Pgm*-100. Moreover the rare *Pgm*-70 was scored in both species. The predominant *Pgm*-70 and the rare *Pgm*-40 and -50 were present in *N. bryope*. In the species with polymorphism at this locus, this enzyme showed a single banded or two banded electrophoretic pattern, though no homozygous individuals for the rare alleles was observed. This indicates that this enzyme system for five species of Japanese *Neoclinus* has a monomeric structure as known for many other fish species (Utter and Hodgins, 1970, 1972).

Phosphomannose isomerase (*PMI*; Fig. 2M): Two alleles were present at this monomeric en-

Table 3. Gene frequencies at 19 loci in 5 Japanese species of *Neoclinus* from Shirahama, Nb, *N. bryope*; Nc, *N. chihiroe*; No, *N. okazakii*; Nl, *N. lacunicola*; Nt, *N. toshimaensis*. The numbers of fish examined are given in parentheses. * Significant deviations from Hardy-Weinberg distributions (χ^2 goodness of fit, $p<0.05$)

Locus	Allele	Nb (41)	Nc (27)	No (12)	Nl (64)	Nt (40)
AAT-1		1.000	1.000	1.000	1.000	1.000
AAT-2	65	0.017	0.063			
	80	0.966	0.906			
	100	0.017	0.031	0.643	1.000	1.000
	110			0.357		
ACON	80	1.000				1.000
	100		1.000	1.000	1.000	
AK		1.000	1.000	1.000	1.000	1.000
CK	90		1.000			
	100	1.000			1.000	1.000
	110			1.000		
β-GAL		1.000	1.000	1.000	1.000	1.000
Pep-GL	70					0.325
	80					0.675
	90	0.463	0.037		0.367	
	100	0.538	0.963	0.792	0.623	
	110			0.208		
IDH-1	85	1.000				1.000
	100		1.000	1.000	1.000	
IDH-2	70				0.008	0.013
	100	1.000	1.000	1.000	0.976	0.988
	130				0.016	
LDH-1		1.000	1.000	1.000	1.000	1.000
LDH-2	70	1.000	0.133			0.013
	100				1.000	0.988
	115		0.867	1.000		
LDH-3		1.000	1.000	1.000	1.000	1.000
Pep-LGG	85	1.000				
	100		1.000	1.000	1.000	1.000
MDH-1	70	1.000	1.000	0.857		
	100				0.968*	0.988
	120				0.024	0.013
	210			0.142		
	270				0.008	
MDH-2	100				0.969	1.000
	105	1.000	1.000	1.000		
	110				0.031	
6-PGD	80	0.017				
	95				0.008	
	100	0.983			0.969	1.000
	110				0.023	
	120		1.000	1.000		
PGM	40	0.034				
	50	0.017				
	70	0.948	0.067		0.031	
	100		0.933	1.000	0.969	1.000
PMI	90			0.167	0.017	
	100	1.000	1.000	0.833	0.983	1.000
SOD	30					0.600
	80		0.056			
	100				1.000	0.400
	160	0.981	0.611	1.000		
	240	0.019	0.333			

zyme. The allele *Pmi*-100 was common among all five species. *N. toshimaensis*, *N. bryope* and *N. chihiroe* were monomorphic for the allele, while *N. lacunicola* and *N. okazakii* possessed rarely another allele *Pmi*-90. In the latter two species, single banded or two banded electromorphs were observed.

Superoxide dismutase (*SOD*; Fig. 2N): Five alleles were scored in this single locus system. *N. lacunicola* was monomorphic for allele *Sod*-100. *N. toshimaensis* had, besides the *Sod*-100, a unique *Sod*-30 which is predominant. *N. okazakii* was fixed for *Sod*-160. The *Sod*-160 was also predominant for and common between *N. bryope* and *N. chihiroe*. Besides, an allele *Sod*-240 was common between these two species, though the gene frequency of the former was fairly lower than that of the latter species. Further, *N. chihiroe* had another rare *Sod*-80.

The observed heterozygosity (*H ob*) in a species was calculated by directly counting the number of heterozygote at the 19 loci examined. The expected heterozygosity (*H exp*) was calculated as follows: $H=1-\sum_{i}^{n} P_i^2$, where n is the number of alleles at the locus and P_i is the frequency of the i^{th} allele in the population of the species. The mean heterozygosity over all 19 loci provided the value of average heterozygosity (H) for each species. At almost all of the polymorphic loci, excepting the *MDH-1* locus of *N. lacunicola*, the deviation of observed number from the expectation was not significant (assuming Hardy-Weinberg equilibrium; Table 3).

Summary of genetic variablility in the examined *Neoclinus* spp. was shown in Table 4. Proportion of polymorphic loci of *Neoclinus* spp. ranged from 0.211 to 0.368 when a locus was considered polymorphic in populations in which the frequency of the most common allele was less than 0.99. The range of observed and expected average heterozygosities of five species were 4.3 to 7.7% and 3.9 to 6.9%, respectively. The obtained values between observed and expected heterozygosities in each species were almost identical. According to the obtained levels of the average heterozygosities and the proportion of polymorphic loci, these values should be considered to be in the range of the values obtained by other authors in fish species (Nevo, 1978; Nevo et al., 1984). The above definitely supports that the examined five species of Japanese *Neoclinus* consisted of independent Mendelian populations.

Table 5 shows matrices of values of Nei's (1972) genetic distance (D) and the fraction of diagnostic loci in gene-enzyme. Diagnostic loci are those loci for which individuals of a given genotype can be assigned to a species with a 1% possibility of incorrect assignment (Ayala and Powell, 1972). Genetic distance and the fraction of diagnostic loci demonstrate the same relationships.

A phylogenetic tree was constructed from indices of genetic distance according to the unweighted paired-group method (UPGMA; Fig. 3). Crude estimates of divergence time presented by Nei (1975) and Vawter et al. (1980) were also shown in Fig. 3. The UPGMA tree showed that the 5 species of Japanese *Neoclinus* clustered into two major groups. One consists of three mem-

Table 4. Proportion of polymorphic loci and average heterozygosity for examined loci in five Japanese species of *Neoclinus*.

Species	Polymorphic loci	Average heterozygosity	
		observed	expected
N. lacunicola	0.368	0.043	0.042
N. toshimaensis	0.263	0.051	0.052
N. bryope	0.263	0.043	0.039
N. chihiroe	0.263	0.057	0.059
N. okazakii	0.211	0.077	0.069

Table 5. Nei's measure of genetic distance (above diagonal) and fractions of diagnostic loci (below diagonal) in comparison among 5 Japanese species of *Neoclinus*.

Species	Nl	Nt	Nb	Nc	No
N. lacunicola		0.175	0.656	0.450	0.410
N. toshimaensis	3/19		0.500	0.695	0.634
N. bryope	9/19	8/19		0.477	0.576
N. chihiroe	7/19	10/19	7/19		0.113
N. okazakii	6/19	9/19	8/19	2/19	

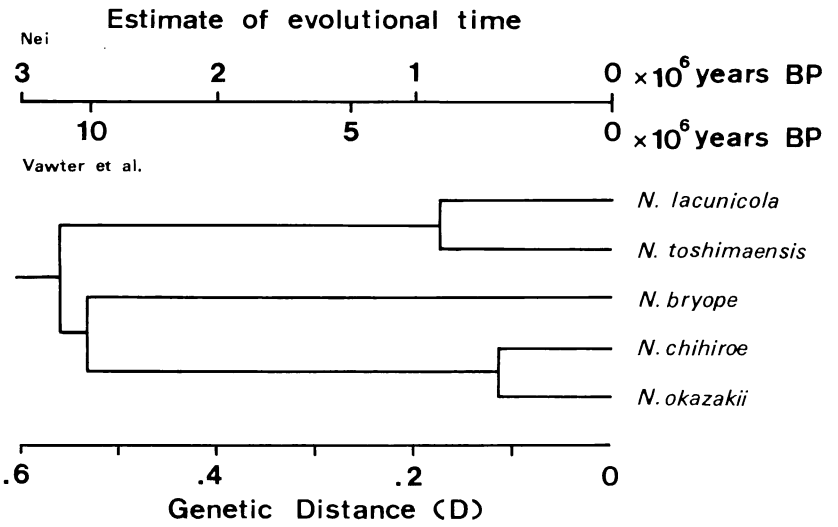


Fig. 3. Phenetic dendrogram produced using UPGMA procedure of cluster analysis on Nei's *D* values given in Table 8, with two different estimates of evolutional time (Nei, 1975 and Vawter et al., 1980).

bers of *N. bryope* complex. Another consists of *N. lacunicola* and *N. toshimaensis*. This agrees with the grouping based on morphology. One group of *N. bryope* species complex, was subdivided into two groups, *N. bryope* and the other two species with an average *D* of 0.527. It is somewhat surprising that, despite of the close similarity in appearance observed in three members of *N. bryope* complex, the distances between *N. bryope* and other two members are fairly large as compared with those between *N. chihiroe* and *N. okazakii*.

Habitat partitioning and overlap. The numbers of fishes collected at each station were presented in Table 6. The percentages of occurrence for each species in each habitat category were shown in Fig. 4.

N. bryope was dominant in TP habitat and they occurred rarely in other three habitats.

N. chihiroe occurred in ME, ME-VE and VE

Table 6. The number of fishes collected at each station. The number of fishes over 40 mm in standard length (adults) are shown in parentheses. Nb, *N. bryope*; Nc, *N. chihiroe*; No, *N. okazakii*; Nl, *N. lacunicola*; Nt, *N. toshimaensis*.

Station (Habitat)	Nb	Nc	No	Nl	Nt	Total
A (TP)	31 (6)					31 (6)
B (ME-VE)			2 (2)			2 (2)
C (ME-VE)	1 (1)					1 (1)
D (ME-VE)	1 (1)	5 (2)	7 (4)	10 (5)		23 (12)
E (ME)	1 (0)	16 (10)	6 (4)	90 (50)		118 (64)
F (ME)		6 (2)				6 (2)
G (TP)	44 (28)		3 (3)			47 (39)
H (VE)			10 (7)		3 (1)	13 (8)
I (VE)	1 (1)		1 (1)	10 (5)	9 (2)	21 (9)
J (VE)	1 (1)	1 (0)	4 (2)	1 (1)	50 (40)	57 (44)
K (VE)	2 (1)	14 (0)	8 (6)	21 (13)	48 (37)	93 (57)
L (VE)				5 (2)	2 (0)	7 (2)
M (TP)	6 (4)					6 (4)
N (VE)	1 (1)		7 (3)		6 (5)	14 (9)
Total	89 (52)	42 (14)	48 (32)	137 (76)	118 (85)	434 (259)

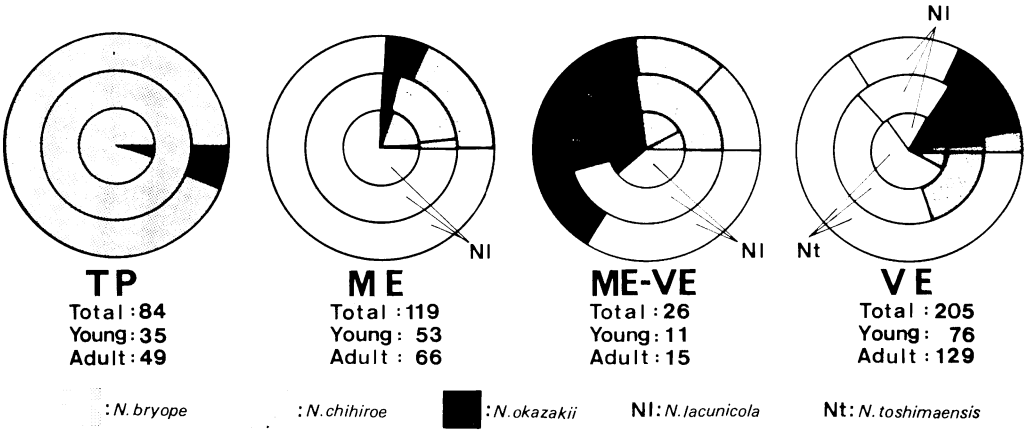


Fig. 4. The percentage occurrences of each species in each habitat category. Inner circle: total; middle doughnut: young (less than 40 mm in SL) only; outer doughnut: adults (over 40 mm in SL) only. The numerals show the number of individuals.

habitats and did not predominate in any of them. Interestingly, all individuals collected in VE habitat were young (less than 40 mm in SL). They do not seem to be able to survive until matured in this habitat. In ME-VE habitat, the percentage of adult of this species was rather low. Since ME-VE habitat is rather particular one and narrowly seen in the waters, moreover, it may not support so many fishes. On the other hand, in ME habitat, this species was subdominant with both young and adults having similar percentage. Thus, the main habitat of *N. chihiroe* is believed to be ME habitat.

N. okazakii occurred in all habitat categories. In TP and ME habitats, the percentages of occurrence of this species were small. The largest percentage of this species was observed in ME-VE habitat. In VE habitat, both young and adults with similar percentage shared the same subdominancy with *N. lacunicola*. Taking into consideration that ME-VE habitat may not support so many fishes, the main habitat of *N. okazakii* is believed to be VE habitat.

N. lacunicola occurred in ME, ME-VE and VE habitats. They were dominant in ME habitat. The percentage of occurrence decreased in ME-VE habitat and further decreased in VE habitat. Their main habitat is believed to be ME habitat.

N. toshimaensis was restricted to VE habitat, where they were dominant.

The three members of *N. bryope* complex partition their main habitat. However, the habitat partitioning among them seems to be imperfect even in the matured individuals. Especially, in ME-VE habitat, the three species intermingled in considerable ratio, though the fishes collected in this habitat were limited. It is worthy to note that in VE habitat, the percentage of occurrence of young of *N. chihiroe* was similar to that of young of *N. okazakii*, while adults of *N. okazakii* occurred in similar percentage with young, adults of *N. chihiroe* were absent. This fact suggests that *N. chihiroe* do not stay away from VE habitat at the recruitment from pelagic life, but that they are eliminated from there as they approach maturing by the selection pressure from the environment. The settlement site selection at the recruitment may not be so rigid in the three members of *N. bryope* complex based on the observation of imperfect habitat partitioning in these three species.

N. lacunicola and *N. toshimaensis*, which constitute another major group, also partition their main habitat. These two species coexisted in VE habitat. Fukao (1980) noted that *N. lacunicola* inhabited only on the cliff (in VE habitat). In the present study, *N. lacunicola* occurred in Sts. I, J, K and L of VE habitat (Table 9). Sts. I and L are cliffed rocky area. St. K has also cliffed faces in the complex topography. In this station, *N. lacunicola* were mostly restricted to cliffed faces. Only 1 individual was collected from St. J, which has no cliffed face. On the other hand, fishes of *N. toshimaensis* collected from cliffed stations, I

and L, were mostly young (less than 40 mm in SL), except for 2 individuals (both with 41.1 mm SL). Fukao (1980) postulated that *N. toshimaensis* prefers turbulent areas in upper subtidal zone and that *N. lacunicola* prefers areas affected by laminar-flow in upper subtidal zone. In the cliffed rocky faces in VE habitat, the wave is laminar-flow rather than turbulent on calm day as in ME habitat, and is turbulent on rough day. It is plausible that these two species select settlement site at the recruitment from pelagic life depending on the status of water movement. It is probably that the fish of *N. toshimaensis* settle on the cliffed faces on rough day and thereafter they may be eliminated from there as they approach maturity. In these two species, thus, the settlement site selection seems to be rigid and the habitat partitioning in matured individuals seems to be nearly perfect.

The main habitat of *N. chihiroe* and of *N. okazakii* overlapped with those of *N. lacunicola* and of *N. toshimaensis*, respectively. *N. lacunicola* predominated over *N. chihiroe* in their overlapped main habitat, ME habitat. In the deeper station F of ME habitat, however, only *N. chihiroe* was observed and collected. *N. chihiroe* seems to extend their habitat to the more deeper sites than *N. lacunicola*. *N. toshimaensis* predominated over *N. okazakii* in their overlapping main habitat, VE habitat. The largest disparity between these two species was observed in the most turbulent station J. Conversely, the balance of these two species reversed in the least turbulent station H of VE habitat. While *N. toshimaensis* is restricted to VE habitat, *N. okazakii* occurred in other habitat, especialy with considerably high percentage in ME-VE habitat. The center of habitat of *N. okazakii* seems to be on the outskirts of the most turbulent area.

In tide pools, fishes (mostly *N. bryope*) were found among sea weeds, under cobbles, or in small rock holes. In the underwater observations at high tide, no fish could be found in depressions of reefs which become tide pools at low tide. This indicates that *N. bryope* may move from pools to upper part of the reef with flowing tide and return to pools at low tide. Some blennies were observed to move with tide along the intertidal reef (Fukao, unpublished data). In ME and ME-VE habitats, most individuals of *N. bryope* complex and all of *N. lacunicola* were found in small rock holes perhaps burrowed by bivalves. Rarely, fishes of *N. bryope* complex were seen to hover on the rocky substrate freely. In VE habitat, fishes of all species were found in empty shells of a barnacle, *Balanus tintinnabulum volcano*, with exceptional individuals of *N. bryope* complex which were seen to hover on the substrate freely. It seems likely that *N. bryope* have rather weak reliance, *N. chihiroe* and *N. okazakii* increasing reliance, and *N. lacunicola* and *N. toshimaensis* the most intense reliance on the hole.

Discussion

N. lacunicola and *N. toshimaensis* are clearly distinct from *N. bryope* complex in replaced alleles for three loci, *MDH-1*, *MDH-2* and *SOD*. The clear distinction between *N. lacunicola* and *N. toshimaensis* is recognized in alleles for *ACON*, *Pep-GL* and *IDH-1* loci. In the same manner, *N. bryope* is clearly distinct from *N. chihiroe* and *N. okazakii* in alleles for *ACON*, *CK*, *IDH-1*, *Pep-LGG* and *6-PGD*. The clear distinction between *N. chihiroe* and *N. okazakii* is also recognized in alleles for *CK*. The above definitely indicate that the introgression is negligible between pairs of the five species of Japanese *Neoclinus* in Shirahama, where they are sympatric. Of the five species, *N. bryope* and *N. okazakii* occurred in other localities. *N. bryope* also occurred in Misaki, Kanagawa Prefecture (Fukao, 1987). *N. okazakii* occurred in Heshikiya, Okinawa Prefecture (Fukao, unpublished data).

A number of studies and reviews demonstrate the crude ranges of estimated genetic distance values betweeen taxa for each taxonomic rank of organisms. In the case of freshwater and marine fishes, for example, the average *D* values at population level, the species level, and at the generic level were 0.05 with a range of 0.002 to 0.065, 0.30 with a range of 0.025 to 0.609, and 0.90 with a range of 0.580 to 1.21, respectively (Shaklee et al., 1982). It has been considered that, despite of considerable variation, the magnitude of genetic distance corresponds generally to the rank of the taxa in many organisms (Nei, 1975; Avise, 1976), with some exceptional cases as observed in primates (King and Wilson, 1975; Nozawa et al., 1977).

Relatively small distances of 0.113 and 0.175 were scored between *N. chihiroe* and *N. okazakii* and between *N. lacunicola* and *N. toshimaensis*, respectively. However, they are sympatric in

Shirahama and well isolated genetically, with clearly distinctive allele between pairs of one or three enzyme loci as noted above. Thus, despite of the smaller *D* values between the pairs, these four forms clearly represent the full species.

Fukao (1980) revised Japanese species of *Neoclinus* and discussed the place of origin of the genus. In the study, the existence of *N. nudus* described by Stephens and Springer (1971) from Taiwan was overlooked and the three species of *N. bryope* complex were treated as one species, *N. bryope*. Now, genus *Neoclinus* contains 3 Californian species and 6 western Pacific species. Thus, the western Pacific forms are superior to the Californian forms in the number of species. However, the origin of the fishes is believed to be waters of the New World as assumed by Hubbs (1952, 1953), Stephens (1961) and Fukao (1980), because chaenopsids, which are considered to be derived from the ancestral stock of *Neoclinus*, are restricted to the New World tropics (Stephens, 1963, 1970) and also because labrisomids some ancestors of which are considered to give rise to *Neoclinus*, are representatives of waters of the New World (Hubbs, 1952; Springer, 1970) and are absent in Japan.

Hubbs (1953) assumed that *N. bryope* emigrated to Japan through the Aleutians during an intergracial period. While, it is well known that a vast array of marine organisms might have emigrated from the temperate western coast of America to the coast of the western Pacific through the northern Pacific during a period from the late Pliocene to the early Pleistocene. This large scale mass directional emigration of marine organisms was well documented by Nishimura (1980). The western Pacific forms of *Neoclinus* or their ancestors might have also emigrated to the coasts of the western Pacific during this period rather than during the intergracial period, because we could not find any significant knowledge supporting the emigration during an intergracial period.

Two crude estimates of divergence time were calculated (Fig. 3), based on the assumption that the genetic distance (*D*) is linearly related to the time after divergence of two populations. Wallis and Beardmore (1984) adopted the estimate of Vawter et al. (1980) for some closely related goby species. However, Nei's estimates agree well with the geological events in the case of the three populations of *Salmo mykiss* Walbaum (Salmonidae; Okazaki, 1984), and the populations of ayu fish, *Plecoglossus altivelis* Temminck and Schlegel (Nishida, 1985). If the estimates of Vawter et al. (1980) are adopted, it can be interpreted from the present results that the fishes of *Neoclinus* emigrate to the coasts of the western Pacific after the completion of speciation events in the coastal waters of the eastern Pacific. According to Nei's estimates, conversely, most of the speciation events might take place during the emigration process oı after the settlement in the coastal waters of the wes tern Pacific. In the present study, we would like to take a view that the speciation events took place in the emigrated coastal waters of the western Pacific, except for the separation between two major groups, based on the following reasons.

From the zoogeographical point of view, most of organisms of the so-called Japan-Oregon elements are considered to have originated in the temperate waters of the western coast of America and to have emigrated to Japan, where the secondary radiation took place (Nishimura, 1980). Nishimura (1980) postulated the conditions that permit the adaptive radiation of organisms as follows: small population of ancestral form invades into a space that has unoccupied ecological niches in a relatively complex environment with high productivity, and then they become isolated from the original stock for a sufficient period.

Two major groups could be recognized in the Japanese *Neoclinus*. The three members of *N. bryope* complex and the two members of another major group, *N. lacunicola* and *N. toshimaensis*, partition their main habitats. These situations could be regarded as the results of a kind of adaptive radiation. Absence of a minimal hypural, the more reduced lateral line and squamation of *N. lacunicola* and *N. toshimaensis* as compared with the three species of *N. bryope* complex, indicate that they may have closer relationship to the chaenopsids (after Rosenblatt and Stephens, 1978 and Fukao, 1980), a group which are possibly better adapted to the tubiculous way of life than *Neoclinus* (Stephens, 1961). In other words, *N. lacunicola* and *N. toshimaensis* are believed to be a more specialized group than *N. bryope* species complex. Now the speciation events could be postulated as follows in connection with the emigration events.

An ancestral founder population of *N. bryope* species complex emigrated to the western Pacific

in the earlier periord of the large scale mass directional emigration event and then differentiated to the present *N. bryope* species complex. *N. bryope* adapted to the rocky intertidal environment with the relatively weak reliance on the holes. *N. chihiroe* and *N. okazakii* adapted to the upper subtidal of the moderately exposed rocky reefs and to that of the very exposed rocky reefs respectively, both with the increasing reliance on the holes.

A common ancestral founder population of *N. lacunicola* and *N. toshimaensis* emigrated to the western Pacific in the later period of the event and then differentiated to the present two species in the temperate waters. *N. lacunicola* adapted to the upper subtidal of the moderately exposed rocky reefs and *N. toshimaensis* to the upper subtidal of the very exposed rocky reefs, both with the intense reliance on the holes. The causal process underlying the speciation events may possibly be accounted by the disruptive selection on habitat preference.

The main habitats of *N. chihiroe* and *N. okazakii* are overlapping with those of *N. lacunicola* and *N. toshimaensis* respectively. In both pairs, the species of the more specialized group predominate in the overlapping main habitats and have the more rigid habitat preference than the two members of *N. bryope* complex. These situations seem to be the result of competition between the species having similar requirement for life. In the competition, the less specialized forms may be displaced from the most suitable sites by the more specialized forms. This idea appears to be consistent with the hypothesis that the ancestral founder population of the more specialized group emigrated later than that of *N. bryope* complex. If the timing of the emigration was the same or the inverse, the seemingly less specialized form of *N. bryope* complex might be difficult to diverge into the present members under the pressure of the specialized forms. It is plausible that the specialized forms with the more rigid habitat preference might invade into a part of the habitats of less specialized forms.

The fact that adaptive radiation is limited to small scale and that it differs from the fishes of Stichaeidae and Agonidae which are considered to be a striking adaptive radiation in the Japan Sea during the same periods (Nishimura, 1980), may be attributed to the limitations of unoccupied niche. The tropical western Pacific is the home of the radiated blenniids and some of the blenniids extended or restricted their distribution to the temperate waters (Fukao, 1985). An adaptive radiation somewhat similar to that of blenniids may be shown by the chaenopsids in the New World tropics where blenniids are scarce (Stephens, 1963, 1970).

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日本産コケギンポ属魚類の分化について

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和歌山県白浜町，京大瀬戸臨海実験所周辺の磯に生息するコケギンポ属 5 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．その結果，同所的に分布するこれら 5 種間では遺伝子の交換が生じていないことが確認され，それぞれは独立種として分化を遂げていることが判明した．さらに，これらは遺伝的な類縁関係からは 2 群に大別された．すなわち，1 群はコケギンポ，シズミイソコケギンポ及びアライソコケギンポからなり，他の 1 群はイワアナコケギンポとトーシマコケギンポからなっており，これらの結果は形態学的なグループ分けとよく一致するものであった．また，これら 5 種の生息場所について観察した結果，前者の群内では不完全な棲み分けがみられ，後者の群内では比較的厳密な棲み分けが認められた．一方，群間ではシズミイソコケギンポとイワアナコケギンポ及びアライソコケギンポとトーシマコケギンポの主生息場所が重複し，それぞれより穴居性の生活に特化したと考えられるイワアナコケギンポとトーシマコケギンポが優占的であった．この生息場所についての知見と遺伝的距離から推定された分化年代に基づき，これら 5 種の種分化について考察を加えた．

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