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Biochemical Differentiation in Japanese Newts, Genus *Cynops* (Salamandridae)

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ABSTRACT—Genetic variation was surveyed in 38 populations of Japanese newts of the genus *Cynops* using starch gel electrophoresis. *C. ensicauda* from the Ryukyu Archipelago was shown to be genetically well differentiated from *C. pyrrhogaster* from the Japanese main islands. Separation of these two forms at the species level is supported. *C. ensicauda* is genetically divided into two groups, each of which corresponds to previously recognized subspecies. On the contrary, protein variation patterns in *C. pyrrhogaster* are not consistent with the previously recognized subspecies or local races. From available geological information, the electrophoretic clock is calibrated at 1D=13-22 MY in Japanese *Cynops*.

INTRODUCTION

Two allopatric newt species of the genus *Cynops* are known from Japan. *C. pyrrhogaster* occurs on the main islands of Honshu, Shikoku and Kyushu, while *C. ensicauda* inhabits the Amami and Okinawa Groups of the Ryukyu Archipelago. A marked geographic variation in external morphology has been detected within each species [1, 2]. Some authors [3, 4] considered morphological variations of these two species to overlap with each other and doubted the specific validity of *C. ensicauda*,treating it as a subspecies of *C. pyrrhogaster*. However, only a few comparative studies have been made between these two species [3, 5], and clarification of the taxonomic relationships of these newts requires an extensive survey of geographic variation in Japanese *Cynops* from many approaches.

Although North American and European newt species belonging to *Taricha*, *Notophthalmus*, and *Triturus* have been studied electrophoretically for the purposes of population genetics, taxonomy, and evolutionary biology [6-9], no comparable studies have been done on Asian newts. Available data indicate that genetic distance values calculated between populations can differentiate named species or subspecies and, therefore, seem to provide rough estimates of the limits of species within the family Salamandridae. Thus, an electrophoretic analysis should be a useful tool for investigating taxonomic problems among Japanese newts. The present study was undertaken mainly in order to understand the amount of genetic differentiation between *C. pyrrhogaster* and *C. ensicauda*, as estimated from an electrophoretic analysis of protein variation. Moreover, based upon geological data, we have derived a calibration for the electrophoretic evolutionary clock in Japanese *Cynops* and compare the value with those proposed previously [10, 11].

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MATERIALS AND METHODS

A total of 610 newts from 23 populations of *Cynops pyrrhogaster* in western Honshu and Kyushu Islands and 15 populations of *C. ensicauda* in three islands in the Amami Group and four in the Okinawa Group, the Ryukyu Archipelago, were analyzed electrophoretically (Fig. 1 and Table 1). We used southwestern populations of *C. pyrrhogaster* for comparison with *C. ensicauda* since they are geographically adjacent to the range covered by *C. ensicauda*.

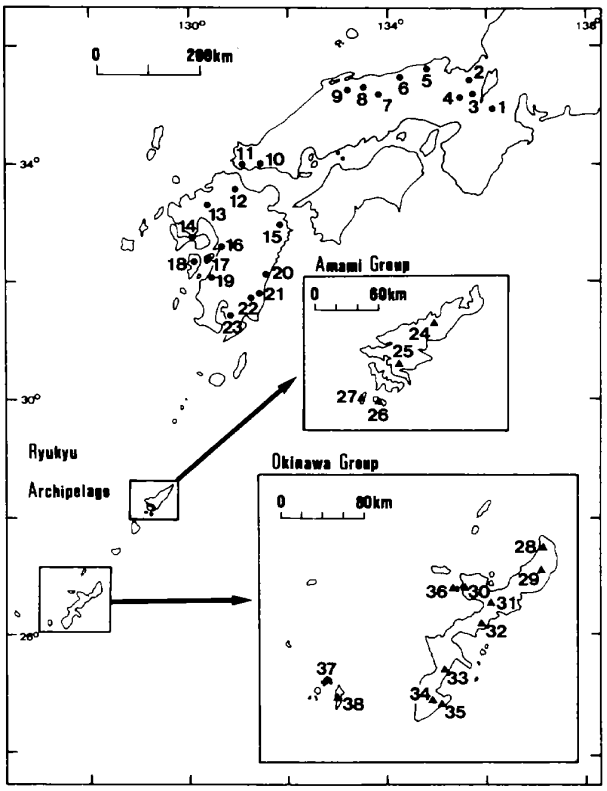

FIG. 1. Geographic localities from which samples of *Cynops* were collected. Localities of *C. pyrrhogaster* are indicated by circles (1–23) and localities of *C. ensicauda* by triangles (24–38).

Samples of liver were removed and maintained frozen at −84℃ until used in electrophoresis. Voucher specimens were fixed in 10% formalin, later preserved in 70% ethanol and deposited in Hayashi's collection at Kyoto University. Homogenized tissue extracts were analyzed by standard horizontal starch gel electrophoresis [12–15], using Connaught starch at a concentration of 11.5%. The enzymes examined and locus designations are listed in Table 2. The buffer system employed in the electrophoretic analysis was 0.155 M tris / 0.043 M citrate, pH 7.0 (1: 15 dilution of electrode buffer for gel) for all enzymes. Genetic interpretations of allozymic data were based on criteria developed by Selander *et al.* [16]. Enzyme nomenclature and E. C. numbers follow the most recent recommendations of the Nomenclature Committee of the International Union of Biochemistry [17] and abbreviations and isozyme designations follow recommendations of Murphy and Crabtree [18]. Electromorphs were designated by letters with "a" representing the most rapidly migrating anodal variant.

The unbiased minimum genetic distance between populations (D) recommended by Nei [19] was computed from observed electromorph frequencies. According to Nei's suggestion [19], all negative values obtained using the collection for small sample sizes were regarded as being equal to 0. A UPGMA phenogram [20] was constructed from genetic distances. A contingency Chi-square test was performed to test for inter-sample electromorph frequency heterogeneity [21]. All samples were also tested for conformance to Hardy-Weinberg expectations with the Chi-square test. For statistical tests, $P<0.05$ was regarded as significant.

RESULTS

A locus was considered polymorphic when two or more electromorphs were detected. Only one of the 15 loci resolved (*Ap-A*) was monomorphic for the same electromorph in all individuals. Table 3 summarizes electromorph frequencies for the remaining 14 polymorphic loci. Fixed differences between *C. pyrrhogaster* and *C. ensicauda* were identified at three loci (*Acp-A*, *Iddh-A* and *M-Me-A*).

Ten of the remaining 11 loci showed significant heterogeneity in electromorph frequencies (Table 4). At four of these 11 loci, a single electromorph predominated in all populations (*Ldh-A*, *Ldh-B*, *M-Mdh-A* and *Pgdh-A*). At *Ldh-A* and *M-Mdh-A* loci, electromorphs other than the common one were unique to single populations. Among the remainder of these four loci, some electromorphs with low to moderate frequency of occurrence were shared among two or more populations. Seven other loci had different variants predominating in different populations (*S-Aat-A*, *Est-1*, *Gpi-A*, *S-Mdh-A*, *S-Me-A*, *Pgm-A*, *S-Sod-A*).

Within *C. pyrrhogaster*, three loci were monomorphic (*Acp-A*, *Ap-A* and *Iddh-A*) and all of 12 polymorphic loci showed statistically significant heterogeneity in electromorph frequencies (Table 4). *C. ensicauda* had six monomorphic loci (*Ap-A*, *Iddh-A*, *Ldh-A*, *M-Mdh-A*, *M-Me-A* and *S-Sod-A*) and significant heterogeneity in electromorph frequencies was observed at seven of nine poly-

TABLE 1. Species, sample size, and locality data for the animals used for electrophoretic analysis

Species	Population number	Locality	N
Cynops pyrrhogaster	1	Shigaraki, Shiga	20
	2	Miyama, Kyoto	39
	3	Kyoto, Kyoto	16
	4	Kameoka, Kyoto	8
	5	Kumihama, Kyoto	4
	6	Tottori, Tottori	19
	7	Ningyo Pass, Okayama	20
	8	Mt. Daisen, Tottori	8
	9	Hirose, Shimane	20
	10	Yamaguchi, Yamaguchi	20
	11	Sanyo, Yamaguchi	20
	12	Yukuhashi, Fukuoka	20
	13	Higashiseburi, Saga	20
	14	Isahaya, Nagasaki	21
	15	Usuki, Oita	5
	16	Shiranui, Kumamoto	20
	17	Kamijima Isl., Amakusa Isls.	18
	18	Shimojima Isl., Amakusa Isls.	20
	19	Minamata, Kumamoto	20
	20	Tsuno, Miyazaki	20
	21	Miyazaki, Miyazaki	17
	22	Tano, Miyazaki	11
	23	Kanoya, Kagoshima	18
Cynops ensicauda	24	Naze, Amami-Oshima Isl.	27
	25	Mt. Kochi, Amami-Oshima Isl.	20
	26	Ukejima Isl.	9
	27	Yorojima Isl.	21
	28	Kayauchibanta, Okinawajima Isl.	5
	29	Mt. Yonaha, Okinawajima Isl.	8
	30	Motobu, Okinawajima Isl.	5
	31	Mt. Nago, Okinawajima Isl.	10
	32	Ginoza, Okinawajima Isl.	10
	33	Nakagusuku, Okinawajima Isl.	18
	34	Tamagusuku, Okinawajima Isl.	10
	35	Chinen, Okinawajima Isl.	9
	36	Sezokojima Isl.	4
	37	Zamamijima Isl.	30
	38	Tokashikijima Isl.	20

TABLE 2. Enzymes and loci analysed in Japanese *Cynops*

Enzyme	Enzyme commission number	Locus
Acid phosphatase	3.1.3.2	*Acp-A*
Aminopeptidase	3.4.11.1	*Ap-A*
Aspartate aminotransferase	2.6.1.1	*S-Aat-A*
Esterase	--	*Est-1*
Glucose phosphate isomerase	5.3.1.9	*Gpi-A*
L-iditol dehydrogenase	1.1.1.14	*Iddh-A*
Lactate dehydrogenase	1.1.1.27	*Ldh-A*
Lactate dehydrogenase	1.1.1.27	*Ldh-B*
Malate dehydrogenase	1.1.1.37	*M-Mdh-A*
Malate dehydrogenase	1.1.1.37	*S-Mdh-A*
"Malic Enzyme"*	1.1.1.40	*M-Me-A*
"Malic Enzyme"*	1.1.1.40	*S-Me-A*
Phosphoglucomutase	5.4.2.2	*Pgm-A*
Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh-A*
Superoxide dismutase	1.15.1.1	*S-Sod-A*

Mitochondrial and supernatant loci are denoted by M- and S- prefixes, respectively.
*NADP-dependent malate dehydrogenase

morphic loci. Among Amami Group populations of *C. ensicauda*, another monomorphic locus was recognized (*Est-1*) and five of eight polymorphic loci were significantly heterogeneous. Among Okinawa Group populations, eight loci were monomorphic and four of seven polymorphic loci showed significant heterogeneity.

The proportions of polymorphic loci ranged from 20.0% (populations 1 and 22) to 53.3% (populations 6, 13, 18 and 19) ($\bar{x}$=37.7%) in *C. pyrrhogaster* and ranged from 13.3% (population 32) to 40.0% (population 33) ($\bar{x}$=27.2%) in *C. ensicauda* (Table 3). The mean number of electromorphs per locus was 1.44 (range 1.20–1.60) in *C. pyrrhogaster*, and 1.32 (range 1.13–1.47) in *C. ensicauda*. The frequencies of genotypes were in good agreement with Hardy-Weinberg proportion in most cases, but the significant heterozygote deficiencies occurred at the *S-Aat-A* locus in one population (population 33), at the *M-Me-A* locus in five populations (populations 12, 13, 16, 19 and 20) and at the *S-Me-A* locus in six populations (populations 2, 7, 8, 9, 14 and 16).

Figure 2 presents a UPGMA phenogram based on the Nei's D values, which are shown in Table 5. The first major dichotomy separates populations of *C. ensicauda* from those of *C. pyrrhogaster* with the mean D value between them being 0.356 (range 0.239–0.724). The mean intraspecific D values are 0.035 (range 0–0.133) in *C. ensicauda* and 0.060 (range 0–0.336) in *C. pyrrhogaster*.

The cluster of *C. ensicauda* is divided into two distinct regional groups, with the mean D value between these two groups being 0.078 (range 0.041–0.133). One subcluster is composed of populations from the Amami Group and another of populations from the Okinawa Group. The mean D values are 0.006 (range 0–0.013) within the former and 0.004 (range 0–0.015) within the latter.

The cluster of *C. pyrrhogaster* is also divided into two distinct groups. One subcluster contains three populations from southernmost part of Kyushu (populations 21–23) and another contains all the remaining populations. The mean D value is 0.156 (range 0.044–0.336) between these two groups.

The mean D value between populations of *C. ensicauda* and three southernmost populations of *C. pyrrhogaster* is 0.532 (range 0.326–0.724), while

TABLE 3. Electromorph frequencies and variability estimates for polymorphic loci in 38 populations of Japanese *Cynops*

Locus	*C. pyrrhogaster*									
	Sasayama race								Hiroshima race	
	1	2	3	4	5	6	7	8	9	10
Acp-A	c	c	c	c	c	c	c	c	c	c
S-Aat-A	b	a(.069) b(.914) d(.017)	a(.107) b(.893)	a(.125) b(.875)	b	b(.974) d(.026)	b	b	a(.139) b(.861)	b
Est-1	b	b	b	b	b	b(.947) c(.053)	b(.947) c(.053)	b	b	b
Gpi-A	c(.975) d(.025)	b(.013) c(.884) d(.103)	b(.063) c(.906) d(.031)	b(.125) c(.750) d(.125)	c	c	c	c	c	a(.150) c(.750) c(.100)
Iddh-A	b	b	b	b	b	b	b	b	b	b
Ldh-A	b	b	b	b	b	b	b	b	b	b
Ldh-B	c	c	c	c	a(.125) c(.875)	a(.233) c(.767)	a(.175) c(.825)	a(.063) c(.937)	c	c
M-Mdh-A	b	b	b	b	b	b	b	b	b	b
S-Mdh-A	c	b(.016) c(.968) d(.016)	c(.937) d(.063)	c(.937) e(.063)	c(.875) d(.125)	a(.026) c(.974)	a(.025) c(.725) d(.250)	c(.438) d(.562)	c	c
M-Me-A	b(.941) c(.059)	b(.677) c(.323)	b(.438) c(.562)	b(.375) c(.625)	b(.375) c(.625)	b(.895) c(.105)	b(.667) c(.333)	b(.875) c(.125)	b(.643) c(.357)	b(.333) c(.667)
S-Me-A	b	a(.219) b(.781)	b	b	b	b	b(.950) c(.050)	b(.750) c(.250)	b(.850) c(.150)	b
Pgm-A	a(.025) b(.500) d(.475)	b(.516) d(.484)	a(.031) b(.375) d(.594)	b(.375) d(.625)	b(.250) d(.750)	a(.053) b(.342) d(.605)	b(.450) d(.550)	b(.313) d(.687)	a(.025) b(.950) d(.025)	a(.150) b(.775) d(.075)
Pgdh-A	b	b	b	b	b	b(.972) c(.028)	a(.025) b(.975)	b b	b(.900) c(.100)	b
S-Sod-A	b	b	b	b	b	b	b	b	b	a(.150) b(.850)
% loci polymorphic	20.0	40.0	33.3	33.3	26.7	53.3	46.7	33.3	33.3	26.7
number of alleles per locus	1.27	1.60	1.47	1.40	1.27	1.60	1.53	1.33	1.40	1.40
Mean heterozygosity	.041	.083	.106	.108	.117	.113	.093	.075	.060	.085

TABLE 3. Continued

Locus	*C. pyrrhogaster* Hiroshima race									
	11	12	13	14	15	16	17	18	19	20
Acp-A	c	c	c	c	c	c	c	c	c	c
S-Aat-A	b	b(.853) c(.147)	a(.050) b(.950)	b(.950) e(.050)	b	b(.775) a(.225)	b	a(.025) b(.975)	b(.944) d(.056)	b(.950) d(.050)
Est-1	a(.025) b(.975)	a(.075) b(.925)	a(.250) b(.750)	b	b(.800) c(.200)	a(.575) b(.425)	a(.306) b(.694)	a(.100) b(.900)	a(.250) b(.750)	b
Gpi-A	a(.100) c(.850) d(.050)	c(.975) d(.025)	b(.025) c(.900) d(.075)	c(.955) d(.045)	c	c	c(.972) d(.028)	b(.425) c(.575)	b(.025) c(.975)	c(.975) d(.025)
Iddh-A	b	b	b	b	b	b	b	b	b	b
Ldh-A	b	a(.075) b(.925)	b	b	b	b	b	b	b	b
Ldh-B	c	c	b(.075) c(.925)	c	c	c	c	c	c	c
M-Mdh-A	b	b	b	a(.050) b(.950)	b	b	b	b	b	b
S-Mdh-A	c	c	c	b(.048) c(.857) d(.095)	b(.300) c(.700)	b(.050) c(.950)	c	b(.025) c(.975)	b(.026) c(.974)	c(.974) d(.026)
M-Me-A	b(.684) c(.316)	b(.472) c(.528)	b(.769) c(.231)	b(.735) c(.265)	b(.500) c(.500)	b(.529) c(.471)	b	b(.971) c(.029)	b(.393) c(.607)	b(.154) c(.846)
S-Me-A	b(.975) c(.025)	b	b	a(.045) b(.955)	b	b(.800) c(.200)	b	b	a(.025) b(.950) c(.025)	b
Pgm-A	a(.050) b(.800) d(.075) e(.075)	b	a(.075) b(.925)	a(.045) b(.955)	b(.800) d(.200)	b	b	a(.025) b(.975)	a(.300) b(.700)	a(.100) b(.900)
Pgdh-A	b	a(.025) b(.950) c(.025)	a(.075) b(.925)	b	a(.100) b(.900)	b	a(.118) b(.882)	a(.125) b(.875)	b	b(.950) c(.050)
S-Sod-A	b(.950) c(.050)	b(.975) c(.025)	b(.750) c(.250)	b	b	b(.725) c(.275)	b(.750) c(.250)	b(.950) c(.050)	b(.900) c(.100)	b
% loci polymorphic	40.0	46.7	53.3	46.7	33.3	40.0	26.7	53.3	53.3	40.0
number of alleles per locus	1.60	1.53	1.60	1.53	1.33	1.40	1.27	1.53	1.60	1.40
mean heterozygosity	.061	.049	.090	.042	.129	.097	.068	.074	.091	.027

TABLE 3. Continued

Locus	*C. pyrrhogaster*			*C. ensicauda*					
	Hiroshima race			Amami Group				Okinawa Group	
	21	22	23	24	25	26	27	28	29
Acp-A	c	c	c	a	a	a(.917) b(.083)	a	a	a
S-Aat-A	b(.722) d(.278)	b(.357) d(.643)	a(.056) b(.500) d(.444)	a(.058) b(.942)	a(.050) b(.900) d(.050)	b(.944) d(.056)	a(.025) b(.975)	a(.100) b(.900)	a(.125) b(.687) d(.188)
Est-1	b	b	b	b	b	b	b	b	b
Gpi-A	b(.971) c(.029)	b(.955) c(.045)	b(.333) c(.667)	c	c	c	b(.029) c(.971)	c	b(.125) c(.875)
Iddh-A	b	b	b	a	a	a	a	a	a
Ldh-A	b	b	b	b	b	b	b	b	b
Ldh-B	c	c	c	b(.096) c(.904)	b(.275) c(.725)	c	c	c	c
M-Mdh-A	b	b	b	b	b	b	b	b	b
S-Mdh-A	c	c	b(.028) c(.972)	d	d(.921) e(.079)	d	d	c(.800) d(.200)	c(.438) d(.562)
M-Me-A	b(.500) c(.500)	c	b(.429) c(.571)	a	a	a	a	a	a
S-Me-A	b	b	b	b	b(.875) c(.125)	b	b	b(.300) c(.700)	b(.313) c(.687)
Pgm-A	a(.500) b(.500)	a(.727) b(.273)	a(.222) b(.750) c(.028)	b(.944) d(.037) e(.019)	b	b(.944) f(.056)	b	b(.625) d(.375)	b
Pgdh-A	b	b	b	a(.042) b(.937) c(.021)	a(.025) b(.975)	a(.333) b(.667)	a(.350) b(.650)	b	b
S-Sod-A	c	c	b(.361) c(.639)	b	b	b	b	b	b
% loci polymorphic	26.7	20.0	40.0	26.7	33.3	28.6	20.0	28.6	28.6
number of alleles per locus	1.27	1.20	1.53	1.40	1.40	1.29	1.20	1.29	1.36
mean heterozygosity	.147	.040	.152	.031	.054	.060	.044	.111	.098

TABLE 3. Continued

Locus	*C. ensicauda* Okinawa Group								
	30	31	32	33	34	35	36	37	38
Acp-A	a	a	a	a	a	a	a	a	a
S-Aat-A	a(.100) b(.800) d(.100)	b(.900) d(.100)	b	b(.972) d(.028)	a(.056) b(.944)	a(.100) b(.900)	b(.750) d(.250)	a(.037) b(.815) d(.148)	b(.833) d(.167)
Est-1	b	b	b	a(.028) b(.944) c(.028)	b	b	b	b	b
Gpi-A	c	b(.050) c(.900) d(.050)	c	c(.861) d(.139)	c	c	b(.125) c(.875)	b(.033) c(.967)	c
Iddh-A	a	a	a	a	a	a	a	a	a
Ldh-A	b	b	b	b	b	b	b	b	b
Ldh-B	c	c	c	c	c	c	c	c	c
M-Mdh-A	b	b	b	b	b	b	b	b	b
S-Mdh-A	c(.500) d(.500)	c(.850) d(.150)	c(.550) d(.450)	c(.719) d(.281)	c(.556) d(.444)	c(.600) d(.350) e(.050)	b(.333) c(.667)	c(.696) d(.304)	c(.550) d(.450)
M-Me-A	a	a	a	a	a	a	a	a	a
S-Me-A	b(.100) c(.900)	b(.450) c(.550)	b(.300) c(.700)	b(.306) c(.694)	b(.278) c(.722)	b(.300) c(.700)	b(.125) c(.875)	b(.117) c(.883)	b(.400) c(.600)
Pgm-A	b	b	b	b	b	b(.950) d(.050)	b	b	b(.950) d(.050)
Pgdh-A	b	b	b	b(.972) c(.028)	b	b	b	b(.981) c(.019)	b
S-Sod-A	b	b	b	b	b	b	b	b	b
% loci polymorphic	21.4	26.7	13.3	40.0	28.6	21.4	28.6	35.7	26.7
number of alleles per locus	1.29	1.33	1.13	1.47	1.36	1.21	1.29	1.43	1.27
mean heterozygosity	.086	.053	.047	.081	.079	.079	.119	.073	.096

TABLE 4. Probability levels of electromorph frequency heterogeneity with a contingency Chi-square test

Locus	Total	*C. Pyrrhogaster*	*C. ensicauda*		
			Total	Amami	Okinawa
Acp-A	.001	—	.05	.05	—
S-Aat-A	.001	.001	NS	NS	.05
Est-1	.001	.001	NS	—	NS
Gpi-A	.001	.001	.001	NS	.01
Iddh-A	.001	—	—	—	—
Ldh-A	.001	.001	—	—	—
Ldh-B	.001	.001	.001	.001	—
M-Mdh-A	NS	.05	—	—	—
S-Mdh-A	.001	.001	.001	.05	.001
M-Me-A	.001	.001	—	—	—
S-Me-A	.001	.001	.001	.01	NS
Pgm-A	.001	.001	.001	NS	.001
Pgdh-A	.001	.001	.001	.001	NS
S-Sod-A	.001	.001	—	—	—

NS indicates statistically insignificant difference at $P<0.05$.

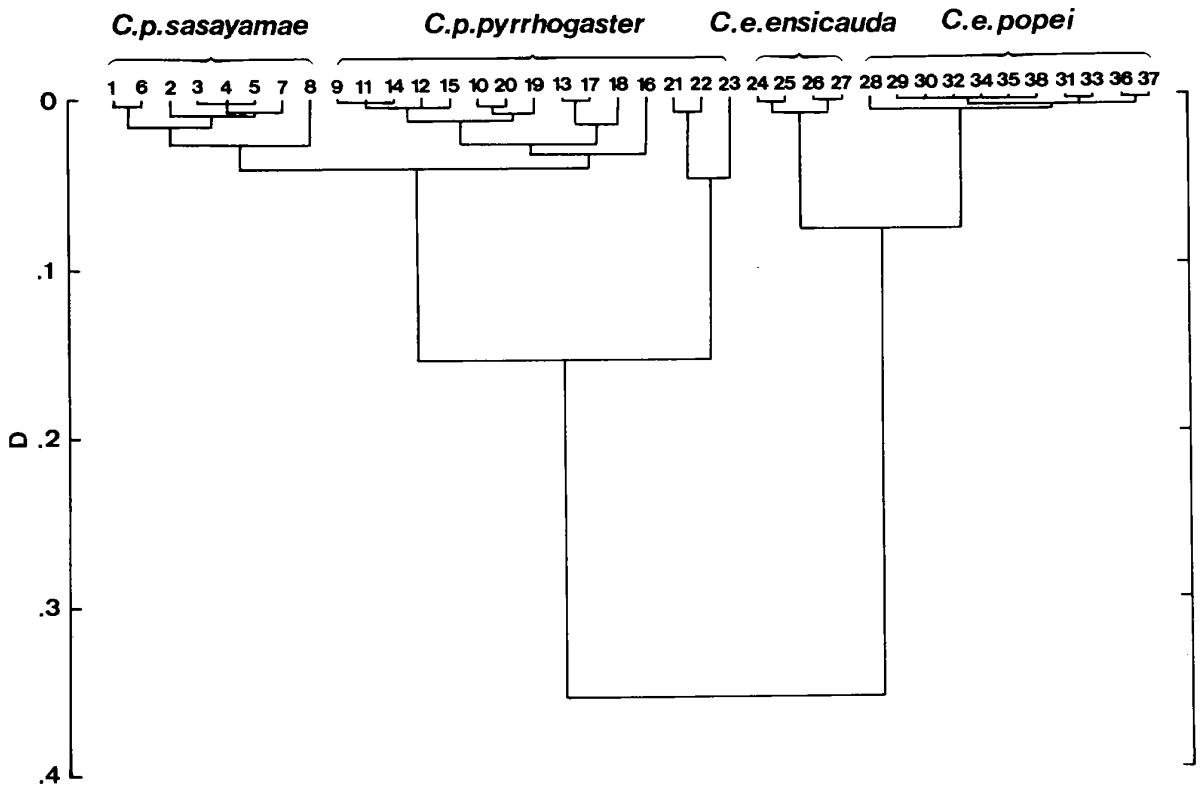

FIG. 2. UPGMA phenogram of populations of Japanese *Cynops* studied. Scale is in genetic distance units of Nei (1978).

TABLE 5. Nei's genetic similarity (below diagonal) and distance (above diagonal) between populations of Japanese *Cynops* studied

	C. pyrrhogaster									
	Sasayama race								Hiroshima race	
	1	2	3	4	5	6	7	8	9	10
1		0.007	0.017	0.024	0.023	0.004	0.010	0.026	0.022	0.038
2	0.993		0.006	0.008	0.011	0.012	0.009	0.029	0.017	0.024
3	0.983	0.994		−0.005	−0.005	0.017	0.007	0.034	0.026	0.018
4	0.977	0.992	1.005		−0.004	0.024	0.011	0.040	0.031	0.017
5	0.977	0.989	1.005	1.004		0.015	0.003	0.028	0.041	0.028
6	0.996	0.988	0.983	0.976	0.985		0.006	0.025	0.035	0.049
7	0.990	0.991	0.993	0.989	0.997	0.994		0.010	0.026	0.032
8	0.974	0.971	0.967	0.961	0.973	0.976	0.990		0.057	0.076
9	0.978	0.984	0.974	0.969	0.960	0.966	0.974	0.944		0.014
10	0.962	0.977	0.982	0.983	0.973	0.952	0.969	0.927	0.986	
11	0.987	0.988	0.980	0.977	0.969	0.975	0.981	0.951	0.997	0.991
12	0.967	0.976	0.973	0.970	0.961	0.954	0.968	0.927	0.997	0.992
13	0.976	0.973	0.961	0.956	0.946	0.963	0.966	0.933	0.990	0.977
14	0.982	0.983	0.970	0.965	0.958	0.968	0.977	0.952	0.998	0.983
15	0.980	0.987	0.988	0.984	0.982	0.972	0.989	0.957	0.994	0.991
16	0.937	0.946	0.938	0.934	0.925	0.925	0.937	0.903	0.969	0.958
17	0.973	0.960	0.939	0.931	0.924	0.957	0.954	0.929	0.979	0.953
18	0.972	0.963	0.945	0.943	0.925	0.955	0.953	0.928	0.978	0.958
19	0.963	0.974	0.978	0.974	0.969	0.955	0.969	0.929	0.986	0.992
20	0.944	0.964	0.974	0.973	0.966	0.934	0.958	0.911	0.983	0.995
21	0.844	0.854	0.860	0.870	0.839	0.833	0.836	0.802	0.853	0.884
22	0.750	0.778	0.805	0.819	0.788	0.746	0.760	0.715	0.775	0.828
23	0.919	0.932	0.936	0.938	0.919	0.908	0.918	0.879	0.945	0.956
24	0.712	0.714	0.719	0.713	0.718	0.698	0.741	0.748	0.732	0.724
25	0.704	0.707	0.709	0.703	0.709	0.691	0.734	0.740	0.729	0.717
26	0.685	0.685	0.690	0.684	0.689	0.668	0.715	0.721	0.709	0.698
27	0.701	0.702	0.707	0.701	0.705	0.685	0.729	0.735	0.725	0.715
28	0.736	0.747	0.742	0.737	0.740	0.724	0.741	0.741	0.751	0.731
29	0.688	0.702	0.692	0.688	0.681	0.667	0.702	0.711	0.732	0.704
30	0.678	0.693	0.680	0.673	0.671	0.658	0.692	0.705	0.725	0.692
31	0.752	0.762	0.752	0.748	0.741	0.734	0.752	0.737	0.787	0.769
32	0.729	0.739	0.729	0.723	0.723	0.711	0.740	0.744	0.765	0.743
33	0.733	0.745	0.733	0.729	0.723	0.715	0.738	0.733	0.770	0.751
34	0.713	0.724	0.714	0.708	0.706	0.694	0.722	0.723	0.751	0.725
35	0.705	0.717	0.706	0.699	0.698	0.685	0.717	0.721	0.746	0.720
36	0.689	0.704	0.688	0.683	0.675	0.668	0.688	0.684	0.735	0.705
37	0.690	0.704	0.690	0.684	0.679	0.670	0.696	0.698	0.736	0.705
38	0.735	0.745	0.737	0.732	0.731	0.718	0.746	0.748	0.768	0.747

TABLE 5. Continued

	C. pyrrhogaster									
	Hiroshima race									
	11	12	13	14	15	16	17	18	19	20
1	0.013	0.034	0.024	0.018	0.020	0.065	0.028	0.029	0.037	0.058
2	0.012	0.025	0.027	0.018	0.013	0.056	0.040	0.037	0.026	0.037
3	0.020	0.027	0.040	0.030	0.012	0.064	0.062	0.057	0.023	0.027
4	0.023	0.030	0.045	0.036	0.016	0.069	0.071	0.058	0.026	0.027
5	0.032	0.040	0.055	0.043	0.018	0.078	0.079	0.078	0.032	0.034
6	0.025	0.047	0.037	0.033	0.028	0.078	0.044	0.046	0.046	0.068
7	0.019	0.033	0.035	0.023	0.011	0.065	0.047	0.048	0.032	0.043
8	0.051	0.075	0.069	0.049	0.043	0.102	0.074	0.075	0.073	0.093
9	0.003	0.003	0.010	0.002	0.006	0.031	0.022	0.023	0.014	0.018
10	0.009	0.008	0.023	0.017	0.009	0.043	0.048	0.042	0.008	0.005
11		0.006	0.006	0.002	0.005	0.035	0.018	0.017	0.012	0.021
12	0.994		0.012	0.007	0.004	0.027	0.028	0.031	0.009	0.008
13	0.994	0.988		0.009	0.014	0.017	0.002	0.015	0.014	0.035
14	0.998	0.993	0.991		0.004	0.037	0.017	0.017	0.017	0.024
15	0.995	0.996	0.986	0.996		0.030	0.028	0.031	0.007	0.010
16	0.966	0.974	0.983	0.964	0.970		0.026	0.054	0.019	0.043
17	0.983	0.972	0.998	0.983	0.972	0.974		0.016	0.034	0.064
18	0.983	0.969	0.985	0.983	0.970	0.947	0.984		0.043	0.062
19	0.989	0.991	0.986	0.983	0.993	0.981	0.966	0.958		0.009
20	0.980	0.992	0.965	0.976	0.990	0.958	0.938	0.940	0.991	
21	0.874	0.858	0.886	0.854	0.849	0.861	0.861	0.891	0.880	0.856
22	0.791	0.791	0.795	0.768	0.779	0.797	0.753	0.784	0.826	0.815
23	0.949	0.951	0.956	0.941	0.941	0.947	0.933	0.935	0.957	0.947
24	0.730	0.733	0.716	0.747	0.747	0.695	0.707	0.707	0.721	0.734
25	0.724	0.728	0.712	0.742	0.740	0.694	0.701	0.701	0.715	0.729
26	0.705	0.710	0.692	0.724	0.727	0.667	0.686	0.686	0.695	0.711
27	0.721	0.726	0.710	0.739	0.742	0.687	0.704	0.706	0.712	0.727
28	0.740	0.734	0.715	0.738	0.739	0.711	0.704	0.703	0.730	0.734
29	0.713	0.717	0.694	0.725	0.715	0.698	0.684	0.692	0.703	0.715
30	0.703	0.705	0.683	0.714	0.704	0.692	0.675	0.674	0.694	0.704
31	0.776	0.778	0.760	0.779	0.771	0.755	0.750	0.753	0.766	0.775
32	0.752	0.753	0.734	0.760	0.753	0.733	0.726	0.725	0.743	0.751
33	0.758	0.758	0.742	0.763	0.755	0.741	0.733	0.734	0.748	0.756
34	0.734	0.736	0.715	0.742	0.735	0.714	0.706	0.705	0.725	0.734
35	0.729	0.731	0.711	0.739	0.730	0.710	0.702	0.701	0.720	0.730
36	0.715	0.717	0.694	0.721	0.718	0.706	0.685	0.693	0.706	0.714
37	0.715	0.717	0.695	0.722	0.711	0.704	0.687	0.688	0.706	0.715
38	0.755	0.757	0.738	0.764	0.758	0.738	0.729	0.728	0.747	0.756

TABLE 5. Continued

	C. pyrrhogaster			*C. ensicauda*						
	Hiroshima race			Amami Group			Okinawa Group			
	21	22	23	24	25	26	27	28	29	30
1	0.169	0.287	0.084	0.339	0.351	0.378	0.355	0.307	0.374	0.388
2	0.158	0.251	0.071	0.337	0.347	0.378	0.353	0.291	0.354	0.366
3	0.151	0.217	0.066	0.330	0.344	0.370	0.347	0.298	0.368	0.386
4	0.139	0.200	0.064	0.338	0.352	0.380	0.355	0.305	0.374	0.396
5	0.176	0.238	0.085	0.331	0.344	0.372	0.350	0.302	0.384	0.399
6	0.183	0.293	0.096	0.359	0.369	0.404	0.379	0.323	0.404	0.418
7	0.179	0.274	0.085	0.300	0.310	0.336	0.316	0.299	0.353	0.368
8	0.220	0.336	0.129	0.291	0.301	0.327	0.309	0.300	0.341	0.350
9	0.159	0.255	0.056	0.312	0.316	0.344	0.321	0.286	0.312	0.322
10	0.123	0.189	0.045	0.323	0.332	0.359	0.335	0.313	0.351	0.368
11	0.135	0.235	0.052	0.315	0.323	0.350	0.327	0.301	0.338	0.353
12	0.153	0.234	0.051	0.310	0.317	0.342	0.320	0.309	0.333	0.349
13	0.121	0.229	0.045	0.333	0.340	0.369	0.342	0.336	0.365	0.381
14	0.157	0.264	0.061	0.291	0.298	0.323	0.302	0.303	0.321	0.337
15	0.163	0.249	0.061	0.292	0.301	0.319	0.299	0.303	0.336	0.352
16	0.150	0.227	0.054	0.363	0.365	0.405	0.376	0.341	0.359	0.368
17	0.150	0.284	0.070	0.346	0.355	0.377	0.350	0.351	0.379	0.393
18	0.115	0.243	0.067	0.346	0.355	0.377	0.348	0.352	0.368	0.394
19	0.128	0.191	0.044	0.327	0.335	0.364	0.340	0.315	0.352	0.366
20	0.155	0.205	0.054	0.309	0.317	0.341	0.319	0.309	0.335	0.351
21		0.009	0.027	0.537	0.551	0.604	0.549	0.544	0.558	0.613
22	0.991		0.069	0.644	0.658	0.723	0.660	0.650	0.657	0.724
23	0.974	0.934		0.397	0.405	0.443	0.411	0.399	0.408	0.444
24	0.585	0.525	0.672		0.003	0.005	0.006	0.093	0.054	0.081
25	0.576	0.518	0.667	0.997		0.013	0.013	0.085	0.043	0.067
26	0.547	0.485	0.642	0.995	0.987		−0.002	0.104	0.062	0.090
27	0.578	0.517	0.663	0.994	0.987	1.002		0.106	0.063	0.091
28	0.580	0.522	0.671	0.911	0.919	0.901	0.900		0.015	0.011
29	0.572	0.518	0.665	0.948	0.958	0.940	0.939	0.985		−0.003
30	0.542	0.485	0.642	0.922	0.936	0.914	0.913	0.989	1.003	
31	0.638	0.578	0.722	0.928	0.936	0.915	0.921	0.995	0.989	0.986
32	0.600	0.537	0.687	0.945	0.900	0.876	0.876	0.989	0.990	0.995
33	0.613	0.550	0.696	0.929	0.917	0.896	0.895	0.992	0.995	1.002
34	0.571	0.507	0.667	0.935	0.960	0.942	0.944	0.992	1.001	0.998
35	0.565	0.500	0.661	0.939	0.949	0.931	0.931	0.993	1.000	1.003
36	0.575	0.521	0.666	0.884	0.945	0.926	0.926	0.998	0.999	1.001
37	0.561	0.503	0.657	0.903	0.954	0.934	0.938	0.992	0.999	1.001
38	0.609	0.555	0.702	0.952	0.938	0.916	0.922	0.994	0.993	0.995

TABLE 5. Continued

	C. ensicauda							
	Okinawa Group							
	31	32	33	34	35	36	37	38
1	0.285	0.316	0.310	0.338	0.349	0.373	0.371	0.308
2	0.272	0.302	0.294	0.323	0.333	0.351	0.350	0.294
3	0.285	0.316	0.311	0.337	0.349	0.374	0.372	0.305
4	0.291	0.324	0.316	0.346	0.358	0.381	0.380	0.313
5	0.300	0.325	0.324	0.348	0.360	0.394	0.387	0.314
6	0.309	0.341	0.336	0.366	0.378	0.403	0.401	0.331
7	0.285	0.300	0.304	0.326	0.333	0.373	0.362	0.292
8	0.305	0.296	0.310	0.325	0.328	0.380	0.359	0.291
9	0.239	0.267	0.261	0.286	0.293	0.308	0.307	0.263
10	0.262	0.297	0.287	0.321	0.328	0.349	0.350	0.292
11	0.253	0.286	0.277	0.309	0.315	0.336	0.336	0.281
12	0.251	0.284	0.277	0.307	0.313	0.333	0.333	0.278
13	0.275	0.309	0.299	0.336	0.342	0.365	0.363	0.304
14	0.249	0.274	0.271	0.298	0.302	0.327	0.326	0.269
15	0.260	0.283	0.281	0.308	0.314	0.331	0.341	0.278
16	0.280	0.310	0.300	0.337	0.342	0.348	0.351	0.303
17	0.287	0.320	0.311	0.348	0.353	0.378	0.375	0.316
18	0.284	0.321	0.309	0.350	0.355	0.367	0.374	0.317
19	0.266	0.298	0.290	0.322	0.329	0.349	0.349	0.291
20	0.256	0.286	0.280	0.309	0.315	0.337	0.336	0.280
21	0.450	0.511	0.489	0.561	0.570	0.554	0.579	0.496
22	0.547	0.622	0.597	0.678	0.693	0.652	0.686	0.589
23	0.326	0.375	0.363	0.405	0.414	0.406	0.421	0.354
24	0.075	0.056	0.074	0.067	0.063	0.123	0.102	0.049
25	0.066	0.047	0.064	0.057	0.053	0.105	0.087	0.041
26	0.088	0.068	0.088	0.076	0.071	0.132	0.110	0.060
27	0.083	0.064	0.082	0.077	0.072	0.133	0.111	0.057
28	0.005	0.008	0.006	0.002	0.007	0.011	0.008	0.008
29	0.011	0.001	0.007	0.001	0.000	0.011	0.005	−0.001
30	0.014	−0.001	0.005	−0.001	−0.003	0.005	−0.002	0.002
31		0.005	0.000	0.003	0.006	0.007	0.008	0.004
32	0.995		0.001	−0.003	−0.004	0.011	0.004	0.000
33	1.000	0.999		−0.001	0.000	0.006	0.004	0.003
34	0.997	1.003	1.001		−0.004	0.007	0.002	−0.001
35	0.994	1.004	1.000	1.004		0.009	0.002	−0.001
36	0.993	0.989	0.994	0.994	0.991		0.000	0.012
37	0.992	0.996	0.996	0.998	0.998	1.000		0.006
38	0.996	1.000	0.997	1.001	1.001	0.989	0.994	

the mean D value between *C. ensicauda* and 20 remaining populations of *C. pyrrhogaster* is 0.330 (range 0.239–0.418). Thus, in spite of their adjacency to the range of *C. ensicauda*, southernmost populations of *C. pyrrhogaster* reach higher level of genetic differentiation from *C. ensicauda* than do remaining conspecific populations.

DISCUSSION

No overlap of electromorphs was detected at three loci (*Acp-A*, *Iddh-A* and *S-Me-A*) between *C. ensicauda* and *C. pyrrhogaster*, and no individual showed any intermediate condition. Furthermore in specimens of *C. pyrrhogaster* from northeastern Japan, we have not detected any individual sharing any electromorph with *C. ensicauda* at the three loci (Hayashi and Matsui, unpublished). Therefore, it is clear that *C. ensicauda* and *C. pyrrhogaster* are genetically distinct from each other.

Levels of genetic differentiation have been investigated at the interspecific and intersubspecific ranks in other genera of the family Salamandridae (*Taricha* [6] and *Triturus* [22]). Genetic differentiation estimates derived from these data might provide an indication of the range in values one might expect between biological species within this family. Nei's D values identified between the two species of Japanese *Cynops* have the range of 0.239 to 0.724 ($\bar{x}$=0.356) and are smaller than interspecific values calculated in *Triturus* species (range from 0.702 to 1.321, $\bar{x}$=1.117), but nearly correspond with the values estimated for three species of *Taricha* (range from 0.261 to 0.687, $\bar{x}$= 0.466). It is also noteworthy that the greatest genetic difference was observed between geographically most adjacent populations of *C. pyrrhogaster* from southernmost Kyushu and *C. ensicauda*. From these genetic view points, two forms of Japanese *Cynops*, one from the main islands (*pyrrhogaster*) and another from the Ryukyu Archipelago (*ensicauda*), are judged to be well differentiated from each other at the species level, and the designation of the latter form as a subspecies of the former is unfounded.

Inger [1] studied morphological variations in newts from Okinawajima and Amami-Oshima Islands, and considered the differences between these two populations sufficient to warrant subspecific distinction. Thus he described the Okinawa population as a distinct subspecies, *Triturus ensicaudus popei*. On the contrary, Koba [23] and Nakamura and Uéno [4], without presenting valid evidence, opposed such a distinction. Our biochemical analysis shows that populations from the Amami Group form a group distinct from those from Okinawa Group with Nei's D values between them ranging from 0.059 to 0.140 ($\bar{x}$=0.084). Although the amount of genetic differentiation between these two groups is smaller than values estimated among intersubspecific populations in *Taricha* (range 0.104–0.309) [24], the geographic pattern of biochemical variation is consistent with the pattern of morphological variations reported by Inger [1], and seems to support the subspecific status of *C. ensicauda popei* (new comb.).

Sawada [2] divided *C. pyrrhogaster* into six "local races" (Hiroshima, Sasayama, Atsumi, Kanto, Tohoku and Intermediate races) from the analysis of the geographic variations in the pattern of ventral markings and body proportions. Later, Mertens [25] gave a name *Triturus* (=*Cynops*) *pyrrhogaster sasayamae* for "Sasayama race" and the other "races" have remained unnamed and included in a single subspecies *C. p. pyrrhogaster*. Our biochemical study contained specimens of two "local races" belonging to different subspecies ("Sasayama race"=*C. p. sasayama*, distributed in northern Kinki and eastern Chugoku Districts (populations 1–8), and parts of "Hiroshima race" =*C. p. pyrrhogaster*, distributed in Kyushu, Shikoku and western Chugoku Districts (populations 9–23)). In the present study, *C. pyrrhogaster* showed significant heterogeneity in electromorph frequencies at all polymorphic loci (Table 4) and it is clear that local populations are genetically well isolated and diverged. Especially, southernmost Kyushu populations (populations 21–23) showed a high level of genetic differentiation from others, and consequently populations of Hiroshima race (i.e. *C. p. pyrrhogaster*) did not form a single group (Fig. 2). This unexpected result indicates that the widely ranging "Hiroshima race" contains at least two distinct groups. By contrast, the "Sasayama race" was found to form a single group.

Our data offer an interesting perspective not only into taxonomic problems but also into genetic divergence in *C. pyrrhogaster*. We are currently conducting an extensive sampling throughout the range of this species and additional electrophoretic studies are in progress.

The molecular clock hypothesis has been applied to date divergence events of taxa [26, 27]. Since molecular clocks need calibrations before applied, some calibrations were estimated for electrophoretic clock, which is a kind of molecular clocks, from different sources. Nei and Roychoudhury [11] originally suggested a calibration of 1D=5 MY irrespective of animal groups. Later, Maxson and Maxson [10] calibrated the electrophoretic clock at 1D=14 MY in plethodontid salamanders based on the correlation with the albumin clock, and the value has generally been used [28, 29]. Calibration could be obtained if geological information of the time of isolation between two populations is available. Because newts cannot cross over the sea, a strait must be a sufficient barrier for gene flow between populations. The formation of the straits between the Japanese main islands and the Ryukyu Archipelago assuredly marked the cessation of gene exchange between populations separated by the sea. The formation of the strait between the Amami and Okinawa Groups also must have prevented newts from gene exchange between isolated populations.

Kizaki and Oshiro [30, 31] estimated that the strait between the mainlands and the Ryukyu Archipelago south of Amami Group was formed about 8 MYBP during late Miocene times and that the strait between the Amami and Okinawa Groups was formed between 1 and 1.5 MYBP during middle Pleistocene times. If the mean D value between *C. ensicauda* and *C. pyrrhogaster* (0.356) corresponds to 8 MY, a calibration of 1D=22 MY is obtained. On the other hand, when the mean D value between populations of the Amami and Okinawa Groups (0.078) is compared to the duration between 1 and 1.5 MY, the electrophoretic clock is calibrated at 1D=13–19 MY. Since both genetic and geological estimates themselves may contain a considerable amount of errors, the difference in these two estimates may not be so great. At least, calibrations of electrophoretic clock in Japanese *Cynops* are regarded as far greater than Nei's original estimate (1D=5 MY) and are more similar to the calibration used in plethodontid salamanders (1D=14 MY). This result seems to suggest the presence of limited range of calibration which is specific to the order Caudata.

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