Isozyme polymorphism and genetic structure of a liverwort *Conocephalum conicum* in natural populations of Japan

Hong Nam Kim¹, Ko Harada^{1,2,†} and Tsuneyuki Yamazaki^{1,2,*}

¹Laboratory of Molecular Population Genetics, Department of Molecular Biology,
Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan

²Laboratory of Population Genetics, Department of Biology, Faculty of Science,
Kyushu University, Fukuoka 812-81, Japan

(Received 3 June 1996)

Haploid liverwort *Conocephalum conicum* is known to be composed of several types (C, A, L, S, J, T and FS). The level of genetic variability of *C. conicum* was surveyed using starch gel electrophoresis at putative 18 enzyme loci from 7 natural populations in Kyushu, Kanto and Tohoku districts. We found two new types which were different not only from either FS or T type, but also from J type in isozyme band patterns, and named them YFS and KYT type. The average heterozygosities of J type from several populations in Kyushu district and Goyosan in Tohoku district were 0.142 and 0.163, respectively. Those of FS and T types from Takaosan in Kanto district, YFS and KYT type from Yakiyama in Kyushu district were 0.146, 0.104, 0.263 and 0.240, respectively. A parameter of genetic differentiation between populations (G_{st}) of the J type were 0.045. This study has confirmed the previous results that the high levels of genetic variability are maintained in natural populations of Kyushu district. High level of genetic variability and low genetic differentiation of Kyushu populations in this haploid plant as well as the data showing high level of G_{st} among the geographically distant populations in various parts of Japan may be explained by the hypothesis that the dominant factor determining the genetic structures of this liverwort is random genetic drift with little migration between different regions of Japan. The finding of various types of *C. conicum* in Japanese populations together with the clear difference in isozyme patterns between types supports the idea that these types are different biological species.

INTRODUCTION

The studies to understand the genetic structure of natural populations and taxonomy of liverwort *Conocephalum conicum* have attracted increased attention (Yamazaki, 1981, 1984; Odrzykoski et al., 1991; Odrzykoski, 1995; Akiyama et al., 1994; Szweykowski et al., 1979, 1981; Toyata, 1994). *C. conicum* is a species with wide geographical distribution, occurring frequently in moist and shaded soil along mountain streams. In both morphological and taxonomical viewpoint, this liverwort has long been treated as one species. It, however, was recognized by Odrzykoski et al. (1991) that *C. conicum* consists of at least five races (forms) with genetically differentiated characteristics. They classified these races as sibling species and named as *C. conicum*-species S, L, A, C and J.

In addition to these five types, the multilocus types, FS and T types have been recognized on the basis of isozyme electrophoretic mobility, morphological difference and habitat preference (Akiyama et al., 1994). Also, chemical types, A, B and C type, have been detected on the basis of phytochemicals in Japan (Toyota, 1994). Among these types, the T and S type were identified as the same type through cross-identification (Odrzykoski, personal communication).

The haploid liverwort *C. conicum* is known to be perennial, dioecious species which reproduces sexually and vegetatively. This species spends most of its life history as a haploid except an ephemeral diplophase. It is generally considered that little genetic variability of haploid plant can be maintained in a natural population, because mutations are directly subjected to natural selection after their occurrence.

However, a large amount of genetic variation was found by Yamazaki (1981, 1984) in natural populations of *C.*

* Corresponding author.

† Present address: Department of Forest Resources, College of Agriculture, Ehime University, Matsuyama, 790, Japan.

conicum in Kyushu district, Japan. And this variation was explained by the balance between accumulation of selectively neutral mutations and elimination by random drift due to finite population size. However, some contradictory findings were soon reported showing that the amount of genetic variation of *C. conicum* was much less in some European populations (see review; Stoneburner et al., 1991) and Honshu of Japan (Akiyama et al., 1994). Wyatt et al. (1989) explained those results by the bottlenecks occurred during glacial period, and also indicated that Yamazaki's results were possibly obtained by the use of small number of loci examined (11 loci) and a large number of nonspecific enzymes (peroxidase, esterase, tetrazolium oxidase). It was also suggested that Yamazaki might have mixed several different types (Akiyama et al., 1994).

Although it has now become clear that *C. conicum* in Europe and Japan was of different types as suggested by Yamazaki (1984), contradictory results obtained by different types have remained to be solved. We therefore reexamined the level of genetic variability and the population structure of *C. conicum* in Kyushu district where Yamazaki carried out his original study, using more loci than the original survey. We report that the high level of genetic variability is indeed maintained in natural populations of *C. conicum* in Kyushu district in Japan, and that

there is a large genetic differentiation among different populations of a type in various regions of Japan as well as different types. Evolutionary implications of these results are also discussed.

MATERIALS AND METHODS

For isozyme study, *C. conicum* was collected from five populations near Fukuoka city in Kyushu district, and from two populations of Goyosan in Tohoku district and Takaosan in Kanto district for reference populations in Japan. Locations of the populations sampled are given in Fig.1 and Table 1. The three types were collected from only Yakiyama population. Because the possibility that the same carpet is composed of genetically identical individuals is very high, we collected only one individual per carpet at intervals of at least 20 meters. As soon as the samples were collected, they were transported immediately to the laboratory and stored in the refrigerator until electrophoresis was carried out.

Enzyme extraction and electrophoresis was conducted following the method of Gottlieb (1981) and Soltis et al. (1983). The samples were incubated in light phase for at least 4 hours before enzyme extraction. A fresh lobe of a thallus was mixed with sea sand in about 1 ml of extraction buffer (0.1M Tris-HCl, pH7.5, 30mM 2-mercapto-

Figure 1. Locations of the populations sampled. Types are indicated in parentheses

Table 1. Populations surveyed, their abbreviations (Abbr.), types and sample size

Population	Abbr.	Type	sample size
Kyushu District			
Yakiyama	YA	J, YFS, KYT	25, 11, 14, respectively
Sefurisan	SE	J	25
Tachibanayama	TA	J	23
Senkokukyo	SEN	J	17
Fukuchiyama	FU	J	23
Tohoku District			
Goyosan	GO	J	9
Kanto District			
Takaosan	TAK	FS, T	4, 12, respectively

ethanol, 1mM EDTA-3Na salt, 10mM MgCl₂, 10mM KCl, 0.1% Triton X-100, and 10mg polyvinylpyrrolidone), and was ground well with a mortar and pestle cooled by ice. The crushed extract was absorbed onto 4 × 15mm paper wicks cut from Whatmann's 3MM filter paper, and then was embedded in starch gel. For electrophoresis, two buffer systems were used: Soltis #10; Tris-EDTA-boric acid, pH 8.6 for electrode buffer, dilution 1:4 of electrode buffer for gel buffer. Soltis #9; L-Histidine-citric acid, pH6.0 for electrode buffer, dilution 1:3 of electrode buffer for gel buffer. Electrophoresis was run with 300V, 90mA using 12% starch gel at 4°C during 4 and 3 h for Soltis #10 and #9 buffer system, respectively.

Recipes for enzyme staining followed the protocol described by Vallejos (1983). The enzymes analyzed, their abbreviations, the buffer systems employed, and number of loci scored are given in Table 2. Putative loci were numbered sequentially from the most anodic to the most cathodic bands as 1, 2, and so on. The most common allele of J type was designated with 1 and all the other alleles were numbered according to the relative rate of mobility to allele 1.

Allele frequency, percentage of polymorphic loci, average number of alleles per locus, average number of alleles per polymorphic locus and average heterozygosity or gene diversity (Nei, 1973) were calculated for all the popula-

tions. We also estimated the H_t (a measure of the total genetic variation), H_s (genetic variation within population) and D_{st} (genetic variation among the population) according to the method of Nei (1973). The coefficient of gene differentiation for interpopulation gene diversity was calculated by the formula, $G_{st} = D_{st}/H_t$. The genetic distance (D) was estimated by $D = -\ln I$, where I is the mean genetic identity, and was calculated by the formula, $I = I_{XY}/\sqrt{(I_x I_y)}$, where I_{XY} , I_x and I_y are the arithmetic means, over all loci of $\sum x_i y_i$, $\sum x_i^2$ and $\sum y_i^2$, respectively (Nei, 1972).

The variants of *C. conicum* were called as sibling species (Odrzykoski et al., 1991), genotype (Akiyama et al., 1994), variation (Suire et al., 1981) and geographic race (Markham et al., 1976). We used a term "type" to avoid confusion of nomenclature among several variants of these species. Types which show genetic difference from one another, mean individual groups distinguishable by morphological, ecological, geographic and chemical differences, and isozyme mobility.

RESULT

Eighteen putative gene loci of 10 isozymes were analyzed in *C. conicum* from 7 populations of Kyushu, Tohoku and Kanto districts in Japan (see Appendix).

We found two distinctive types from Yakiyama popula-

Table 2. Enzymes, their abbreviations (Abbr.), Enzyme Commission numbers (EC), number of loci scored (Scor.), and buffer systems used for electrophoresis

	Enzyme abbr.	EC no.	Scor.loci	Buffer
Isocitrate dehydrogenase	IDH	1.1.1.42	1	Soltis #9
Glutamate Oxaloacetate Transaminase	GOT	2.6.1.1	2	Soltis #10
Esterase	EST	3.1.1.1 or 3.1.1.2	2	Soltis #10
Peroxidase	PO	1.11.1.7	4	Soltis #10
Phosphoglucose Isomerase	PGI	5.3.1.9	1	Soltis #10
Malate dehydrogenase	MDH	1.1.1.37	3	Soltis #9
Aconitase	ACN	4.2.1.3	1	Soltis #9
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	1	Soltis #10
Malic enzyme	ME	1.1.1.40	1	Soltis #10
Glutamate dehydrogenase	GDH	1.4.1.2	2	Soltis #10

Figure 2. Band patterns of common alleles in 5 types of *C.conicum* for GDH, EST and 6-PGD. Note the difference of eletrophoretic mobility rate at each locus.

tion on the basis of electrophoretic mobility rate of common alleles (Fig. 2, Table 3). They indicated unusual isozyme band patterns which are distinctive from each other. They also were distinguished from both FS and T types found by Akiyama et al. (1994) in Japan. We named them KYT and YFS, though they didn't show notable morphological difference from T and FS types, respectively. The type from Goyosan population also showed the distinctive band patterns from the J type of other populations at common alleles of IDH, PO-4, PGI and GDH-1 loci. Such difference wasn't detected among the J types from Kyushu district populations. Because the subtle difference of common alleles among the same types was detected in the previous study (Akiyama et al., 1994), it was treated as J type.

The allelic frequencies, average heterozygosity and other parameters of genetic variability are summarized at Appendix and Table 4, 5 and 6. Two putative loci, po-1 and 3 were monomorphic throughout the populations (Table 5). The J type was very polymorphic at GOT-1, GOT-2, MDH-3 and GDH-1 loci, and other types showed

Table 3. Relative mobility of common alleles for 5 types of *Conocephalum conicum*

	J	GO(J)	YFS	FS	KYT	T
IDH	1.00	1.05	1.00	1.00	1.09	0.94
GOT-1	1.00	1.00	0.89	0.89	0.97	0.94
GOT-2	1.00	1.00	0.94	0.94	1.05	0.87
EST-1	1.00	1.00	1.05	1.00	1.05	0.93
EST-2	1.00	1.00	0.91	0.85	0.91	null
PO-1	1.00	1.00	1.00	1.00	1.00	1.00
PO-2	1.00	1.00	1.00	1.00	1.00	1.00
PO-3	1.00	1.00	0.95	0.95	0.85	0.85
PO-4	1.00	1.17	0.57	0.57	1.57	1.46
PGI	1.00	1.10	1.06	1.00	1.10	1.00
MDH-1	1.00	1.00	0.92	1.00	0.92	1.00
MDH-2	1.00	1.00	1.00	1.00	1.00	1.00
MDH-3	1.00	1.00	0.85	1.00	0.85	1.00, 0.85
ACN	1.00	1.00	1.00	1.00	1.00	1.00
6-PGD	1.00	1.00	0.89	0.89	1.00	1.00
ME	1.00	1.00	1.06	1.00	1.00	1.00
GDH-1	1.00	0.90	0.89	0.89	0.74	1.15
GDH-2	1.00	1.00	0.57	1.00	0.57	null

The J indicates J type from populations of Kyushu district only.

Table 4. General parameters of genetic variability

	Average number of alleles per locus	Average number of alleles per polymorphic locus	Percentage of polymorphic loci
YA(J)	1.667	2.500	0.444
SE(J)	1.611	2.222	0.500
TA(J)	1.500	2.500	0.333
SEN(J)	1.944	2.417	0.667
FU(J)	1.611	2.333	0.500
total ^(a)	2.944	3.188	0.889
YA(YFS)	1.722	2.182	0.611
YA(KYT)	1.611	2.000	0.611
total ^(b)	4.167	4.353	0.944
GO(J)	1.556	2.250	0.444
TAK(FS)	1.389	2.000	0.500
TAK(T)	1.333	2.500	0.222
total ^(c)	4.833	5.059	0.940
average ^(d)	1.648	2.370	0.481

^(a) total value of J type from Kyushu district.

^(b) total value of YFS, KYT and J type from Kyushu district.

^(c) total value of the whole type from Kyushu, Tohoku and Kanto districts.

^(d) average value of J type from the whole population.

various patterns in the extent of polymorphism per locus.

The average number of alleles per locus showing the genetic richness of the population varied from 1.333 for the Takaosan (T) population to 1.944 for the Senkokukyo (J) population (Table 4). In general, the most notable richness in the number of alleles was recorded for the GOT-1, GOT-2, GDH-1 and GDH-2 loci (Appendix). The percentage of polymorphic loci observed varied from 0.222 for Takaosan (T) population to 0.667 for Senkokukyo (J).

We analyzed the average heterozygosity per locus from all the populations and types (Table 5). In all the loci studied, the average heterozygosity per population was high. The average heterozygosity of J type from all the populations and from those except Goyosan were 0.145 and 0.142, respectively. From Yakiyama population, if we distinguish our types, the values of the average heterozygosity are 0.134, 0.263 and 0.240 for J, YFS and KYT types, respectively. Pooling (or ignoring) our types (YFS and KYT), this value was 0.447.

We calculated the ratio of diversity among populations to the total diversity (G_{st}) on the basis of 18 loci analyzed (Table 6). G_{st} value of J type from the whole population and from that except Goyosan were 0.154 and 0.045, respectively.

DISCUSSION

Genetic diversity within and between populations. Several parameters relevant to genetic variation in natural populations were estimated in several types of *C. conicum* (Tables 4 and 5). Unweighted averages of

these parameters, percentage of polymorphic loci per population (Pp) and number of alleles per locus per population (Ap) were 0.481 and 1.648, respectively. The parameter values estimated in this study are much higher than those in most liverwort studies. The lowest values were obtained in *Plagiochila porelloides* (Pp = 0.00, Ap = 1.00., see review; Stoneburner et al., 1991). Similar low values were also reported in S type of *C. conicum* (Pp = 0.044, Ap = 1.04, see review; Stoneburner et al., 1991). Relatively high values were reported in three different types of *C. conicum* including J types, which were collected in Kansai district in Japan (Pp = 0.361, Ap = 1.415) (Akiyama et al., 1994). The highest estimate thus far were reported by Yamazaki (1984) using J type collected from several populations in Kyushu district (Pp = 0.827, Ap = 2.81). A part of rather large differences in these parameters among different studies may have arisen due to the difference in the sample sizes since both the number of alleles and proportion of polymorphic loci are very sensitive to the differences in sample size.

Considering the high values obtained in both previous studies and also this study, it is certain that these population parameters in at least J type of *C. conicum* are high, namely the J type is much more polymorphic than others previously studied.

Heterozygosity or gene diversity is the best parameter which describes the amount of genetic variability in natural populations without being affected by sample size, though it is actually the expected heterozygosity in the case of *C. conicum* since liverwort is a haploid plant.

In liverwort, Yamazaki (1981, 1984) reported the highest level of genetic variations. Among several types of *C. conicum*, the average heterozygosities of J type were 0.167, 0.080 and 0.083 for Kyushu (Yamazaki, 1984), Kansai and Kanto districts (Akiyama et al., 1994), respectively. The values of 0.113 and 0.107 were obtained in T(S) and FS types from Kanto districts (Akiyama et al., 1994), in contrast to much lower heterozygosities, 0.012, 0.025 and 0.044 in S, L and A types in Europe and North American populations, respectively (see review; Stoneburner et al., 1991).

In this study, the estimated average heterozygosity of J type in Kyushu populations was 0.142, which is very similar to that of Yamazaki (1981, 1984) sampled from the same population. The values of our YFS and KYT types were also high, 0.263 and 0.240, respectively. Akiyama et al. (1994) pointed out that Yamazaki might have mixed the several types. However, if we estimate the average heterozygosity by simply mixing the above three types together, the heterozygosity is 0.447 (see Table 5), much higher than the estimate reported by Yamazaki. Wyatt et al. (1989) also argued that the high heterozygosity by Yamazaki was due to nonspecific enzymes, peroxidase, tetrazolium oxidase and esterase. We also surveyed peroxidase and esterase. The high level of heterozygosity in

Table 5. Average heterozygosity for each locus and population

	YA(J)	SE(J)	TA(J)	SEN(J)	HU(J)	GO(J)	average ⁽¹⁾ S.E.	average ⁽²⁾ S.E.	YA(YFS)	YA(KYT)	TAK(FS)	TAK(T)	YA(total) ⁽³⁾
IDH	0.211	0	0	0.360	0	0.494	0.178	0.087	0.114	0.074	0	0	0.482
GOT-1	0.320	0.563	0.363	0.594	0.234	0.345	0.403	0.058	0.415	0.070	0.408	0	0.677
GOT-2	0.346	0.493	0.326	0.304	0.234	0.568	0.379	0.051	0.341	0.043	0.49	0	0.507
EST-1	0	0.077	0	0	0	0	0.013	0.013	0.015	0.015	0.627	0	0.441
EST-2	0.422	0.147	0	0.111	0.082	0	0.127	0.064	0.152	0.072	0.298	0	0.706
PO-1	0	0	0	0	0	0	0	0	0	0	0	0	0
PO-2	0.077	0	0	0	0.226	0	0.051	0.038	0.061	0.044	0	0	0.039
PO-3	0	0	0	0	0	0	0	0	0	0	0	0	0.623
PO-4	0	0	0	0.111	0	0	0.019	0.019	0.022	0.022	0	0	0.500
PGI	0	0	0	0.304	0	0	0.051	0.051	0.061	0.061	0	0.527	0.642
MDH-1	0	0.320	0	0.290	0	0.198	0.135	0.062	0.122	0.075	0.397	0	0.601
MDH-2	0	0	0	0	0	0.345	0.058	0.058	0	0.298	0	0	0.077
MDH-3	0.480	0.499	0.234	0.380	0.082	0.197	0.312	0.069	0.335	0.079	0.496	0.624	0.499
ACN	0	0.077	0	0	0	0	0.013	0.013	0.015	0.015	0	0	0
6-PGD	0	0	0	0.290	0.082	0.345	0.120	0.064	0.074	0.056	0.397	0.570	0.449
ME	0.077	0	0.320	0.208	0.082	0	0.115	0.052	0.137	0.057	0.397	0	0.394
GDH-1	0.474	0.442	0.635	0.630	0.320	0.444	0.491	0.050	0.500	0.060	0.43	0.152	0.783
GDH-2	0	0.148	0.159	0.290	0.320	0	0.153	0.056	0.183	0.057	0.496	0	0.621
average	0.134	0.154	0.113	0.215	0.092	0.163	0.145	0.018	0.142	0.021	0.263	0.104	0.447
S.E.	0.044	0.049	0.044	0.048	0.028	0.049				0.054	0.044	0.052	0.059

⁽¹⁾ average heterozygosity of J type from the whole population.⁽²⁾ average heterozygosity of J type from the whole population excluding Goyosan population.⁽³⁾ total average heterozygosity of pooled J, YFS and KYT type from Yakiyama population.

Table 6. Relative measure of genetic diversity for J type from 6 populations of *Conocephalum conicum*. genetic variation within the population (H_s), genetic variation among the population (D_{st}), a measure of the total genetic variation (H_t) and coefficient of gene differentiation (G_{st})

	H_s	D_{st}	H_t	G_{st}
IDH	0.114	0.009	0.123	0.072(0.333)
GOT-1	0.415	0.026	0.441	0.059(0.053)
GOT-2	0.340	0.020	0.360	0.055(0.068)
EST-1	0.015	0.0003	0.016	0.016(0.017)
EST-2	0.153	0.010	0.163	0.063(0.068)
PO-1	0.000	0.000	0.000	0.000(0.000)
PO-2	0.061	0.003	0.063	0.043(0.046)
PO-3	0.000	0.000	0.000	0.000(0.000)
PO-4	0.022	0.001	0.023	0.024(0.882)
PGI	0.061	0.004	0.065	0.060(0.731)
MDH-1	0.122	0.010	0.132	0.075(0.062)
MDH-2	0.000	0.000	0.000	0.000(0.106)
MDH-3	0.335	0.032	0.368	0.088(0.094)
ACN	0.015	0.0003	0.016	0.016(0.017)
6-PGD	0.074	0.005	0.079	0.059(0.075)
ME	0.137	0.007	0.144	0.048(0.053)
GDH-1	0.500	0.040	0.540	0.074(0.116)
GDH-2	0.183	0.009	0.193	0.049(0.055)
mean	0.142	0.010	0.151	0.045(0.154)
S.E.	0.036	0.003	0.039	0.007(0.059)

Values in parentheses are G_{st} for the samples including the Goyosan sample.

this study was not affected much by these enzymes (see Table 5). These analysis clearly shows that species of *C. conicum* in Japan are indeed more polymorphic than those in Europe.

Comparisons of G_{st} , the coefficient of gene differentiation between populations, give a clearer view of population structure of the liverwort. The G_{st} value including Goyosan from northern Japan was 0.154, while that excluding Goyosan population was 0.045 which is similar to the value, 0.031 obtained by Yamazaki (1981, 1984) from populations in Kyushu. It suggests that there is little genetic differentiation among populations in Kyushu district. The fact that all the common alleles of J type from populations in Kyushu is identical (see Table 4 and appendix) clearly supports the above finding. On the other hand, populations of Kanto and Kansai were much more diverged within each district (0.543 and 0.145, respectively., Akiyama et al., 1994). G_{st} values of other types, S, L, and A in Europe were also high, 0.276, 0.232 and 0.231, respectively (see review, Stoneburner et al., 1991).

Genetic variation originates ultimately through mutations. Level of variation depends on various factors such as random genetic drift, selection, migration and historical events. It is, therefore, not so easy to single out a main factor determining the present population structure including the amount of genetic variation.

Heterozygosity can not be expected in haploid organisms which have no genetic shelter against selection. The

average heterozygosity in *C. conicum* is at least the same level as that (0.156) of diploid plants, which is the average of 113 taxa (Hamrick et al., 1979). G_{st} value was also similar to that of higher plant (0.071, Loveless et al., 1984).

From the isozyme data of *C. conicum* which have relatively high level of heterozygosity without genetic differentiation between populations of the same mountain range such as population in Kyushu district in this study, but with big differences between distant populations within Japan, we infer that random genetic drift of selectively neutral mutations and historical events, coupled with high migration within the same mountain range, but with little genetic exchange between distant populations, may be a major determinant of the genetical structure in natural populations.

Evolutionary significance of types. In bryophyte, several types have been detected using isozyme electrophoresis. Types showing difference in multilocus phenotypes were recognized by Dewey (1989) from morphologically uniform liverwort, *Riccia dictyospora*. Intensive studies have been made in *C. conicum* (Odrzykoski et al., 1991). It has been suggested that genetically, this species is differentiated markedly despite the relatively uniform morphology.

On the other hand, considerable variations are maintained in *C. conicum* with respect to flavonoid, and these different types were considered as geographical races (Markham et al., 1976). Suire et al. (1981) reviewed that there were substantial sex related variations in *C. conicum* on terpenoid, and they suggested this species to be in a state of active evolutionary differentiation without full morphological differentiation. Also, the most recent, three chemical types, A, B and C were found on the basis of phytochemicals differences in Japan (Toyota, 1994).

We also found the new types (YFS and KYT) with the distinctive band patterns in electrophoretic mobility of allozymes in *C. conicum* (see Fig. 2 and Table 3). The new types show distinctive difference on genetic distance (see Table 7). Mixture of bands which suggest sexual mating among these types, were not detected. It is difficult to determine definitely if those types are new species or not, due to lack of sufficient evidence to discriminate these different types. It is inferred that there are little exchange of genetic materials between types even though some of them are in the same geographic regions. It seems that most of these different types are different biological species. Considering subtle difference in morphology and distinctive difference in isozyme patterns among them, we believe that the liverwort *C. conicum* is now in a active stage of evolution which has a large genetic differentiation within population and leads to new biological species.

Table 7. Nei's genetic distance among types

	YA(J)	SE(J)	TA(J)	SEN(J)	HU(J)	GO(J)	YA(YFS)	TAK(FS)	YA(KYT)
SE(J)	0.247								
TA(J)	0.110	0.193							
SEN(J)	0.374	0.480	0.290						
HU(J)	0.238	0.280	0.130	0.272					
GO(J)	0.512	0.620	0.356	0.572	0.522				
YA(YFS)	1.422	1.464	1.032	1.362	1.506	1.508			
TAK(FS)	0.818	0.905	0.652	0.801	0.839	0.876	0.666		
YA(KYT)	1.319	1.350	1.254	1.297	1.396	1.107	1.050	1.301	
TAK(T)	1.118	1.121	0.946	1.153	1.124	0.999	1.557	1.220	1.103

We thank Tachida, H. and Odrzykoski, I. J. for their valuable suggestions. Thanks are also to Masuda for her technical support, and Hiraoka for her help in identification of types. This research was supported in part by a grant from Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

- Akiyama, H. and Hiraoka, T. (1994) Allozyme variability within and divergence among population of the liverwort *Conocephalum conicum* (Marchantiales: Hepaticae) in Japan. *J. Plant Res.* **107**, 307–320.
- Dewey, R. M. (1989) Genetic variation in the liverwort *Riccia dictyospora* (Ricciaceae, Hepaticopsida). *Syst. Bot.* **14**, 155–167.
- Gottlieb, L. D. (1981) Electrophoretic evidence and plantpopulations. *Prog. Phytochem.* **7**, 1–46.
- Hamrick, J. L. (1979) Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Annu. Rev. Ecol. Syst.* **10**, 173–200.
- Loveless, M. D. and J. L. Hamrick (1984) Ecological determinants of genetic structure in plant populations. *Annu. Rev. Ecol. Syst.* **15**, 65–95.
- Markham, K. R., Porter, L. J., Mues, R., Zinsmeister, H. D. and Bert G. B. (1976) Flavonoid variation in the liverwort *Conocephalum conicum*: evidence for geographic races. *Phytochemistry* **15**, 147–150.
- Nei, M. (1972) Genetic distance between populations. *Am. Nat.* **106**, 283–292.
- Nei, M. (1973) Analysis of gene deversity in subdivided Populations. *Proc. Natl. Acad. Sci. USA* **70**, 3321–3323.
- Odrzykoski, I. J. and Szweykowski, J. (1991) Genetic differentiation without concordant morphological divergence in the thallose liverwort *Conocephalum conicum*. *Plant Syst. Evol.* **178**, 135–151.
- Odrzykoski, I. J. (1995) Sibling species of the *Conocephalum conicum* complex (Hepaticae, Conocephalaceae) in Poland: distribution maps and description of an identification method based on isozyme markers. *Fragm. Florist. Geobot.* **40**, 393–404.
- Soltis, D. E., Haufler, C. H., Darrow, D. C. and Gastony, G. J. (1983) Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *Am. Fern J.* **73**, 9–27.
- Stoneburner, A., Wyatt, R. and Odrzykoski, I. J. (1991) Applications of enzyme electrophoresis to bryophyte systematics and population biology. *Adv. Bryol.* **4**, 1–27.
- Suire, C. and Asakawa, Y. (1981) Chemotaxonomy of Bryophytes: a survey. In: *Bryophyte Systematics* (eds.: G. C. S. Clarke and J. G. Duckett), pp.447–477. Academic Press, London.
- Szweykowski, J. and Krzakowa, M. (1979) Variation of four enzyme systems in Polish populations of *Conocephalum conicum* (L.) Dum. (Hepaticae, Marchantiales). *Bull. Acad. Polon. Sci. Ser. Sci. Biol.* **27**, 37–41.
- Szweykowski, J., Odrzykoski, I. J. and Zielinski, R. (1981) Further data on the geographic distribution of two genetically different forms of the liverwort *Conocephalum conicum* (L.) Dum.: the sympatric and allopatric regions. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **28**, 437–449.
- Toyata, M. (1994) Phenolic compounds other than flavonoids from Bryophytes. *J. Hattori Bot. Lab.* **76**, 273–281.
- Vallejos, C. Eduardo (1983) Enzyme activity staining. In: *Isozymes in plant genetics and breeding* (eds.: S. D. Tanksley and T. J. Orton), pp.469–510. Elsevier Science Publishers, Amsterdam.
- Wyatt, R. Odrzykoski, I. J. and Stoneburner, A. (1989) High levels of genetic variability in the haploid moss *Plagiomnium ciliare*. *Evolution* **43**, 1085–1096.
- Yamazaki, T. (1981) Genic variabilities in natural population of haploid plant, *Conocephalum conicum* . I. The amount of heterozygosity. *Jpn. J. Genet.* **56**, 373–383.
- Yamazaki, T. (1984) The amount of polymorphism and genetic differentiarion in natural population of the haploid liverwort *Conocephalum conicum*. *Jpn. J. Genet.* **59**, 133–139.

Locus	sample number		Populations							
Allele	YA(J)	SE(J)	TA(J)	SEN(J)	HU(J)	GO(J)	YA(YFS)	TAK(FS)	YA(KYT)	TAK(T)
IDH	25	25	23	17	23	9	11	4	14	12
1.09	0	0	0	0	0	0.222	0	0	1.000	0
1.05	0	0	0	0	0	0.667	0	0	0	0
1.04	0.120	0	0	0.235	0	0	0	0	0	0
1.00	0.880	1.000	1.000	0.765	1.000	0.111	1.000	0.750	0	0
0.97	0	0	0	0	0	0	0	0.250	0	0
0.94	0	0	0	0	0	0	0	0	0	1.000
0.91	0	0	0	0	0	0	0	0	0	0
Het.	0.211	0	0	0.360	0	0.494	0	0.375	0	0
GOT-1	25	25	21	17	23	9	7	4	7	12
1.14	0	0	0	0.118	0.087	0	0	0	0	0
1.07	0.200	0.400	0.238	0.412	0	0.222	0	0	0	0
1.00	0.800	0.520	0.762	0.471	0.870	0.778	0	0	0	0
0.97	0	0	0	0	0	0	0	0	0.714	0
0.94	0	0	0	0	0	0	0.286	0	0.286	1.000
0.89	0	0	0	0	0	0	0.714	1.000	0	0
null	0	0.080	0	0	0.043	0	0	0	0	0
Het.	0.320	0.563	0.363	0.594	0.234	0.345	0.408	0	0.408	0
GOT-2	25	25	21	17	23	9	7	4	7	12
1.05	0.080	0	0	0.118	0.087	0	0	0	0.571	0
1.00	0.800	0.560	0.810	0.824	0.870	0.556	0.429	0	0.429	0
0.94	0.080	0	0.095	0	0	0	0.571	1.000	0	0
0.87	0	0	0	0	0	0.333	0	0	0	1.000
null	0.040	0.440	0.095	0.059	0.043	0.111	0	0	0	0
Het.	0.346	0.493	0.326	0.304	0.234	0.568	0.490	0	0.490	0
EST-1	25	25	23	17	23	9	11	4	14	12
1.05	0	0.040	0	0	0	0	0.455	0.250	0.571	0
1.00	1.000	0.960	1.000	1.000	1.000	1.000	0.364	0.750	0.429	0
0.93	0	0	0	0	0	0	0.182	0	0	1.000
Het.	0	0.077	0	0	0	0	0.627	0.375	0.490	0
EST-2	25	25	23	17	23	9	11	4	14	12
1.06	0	0.080	0	0	0.043	0	0	0	0	0
1.00	0.720	0.920	1.000	0.941	0.957	1.000	0	0.250	0	0
1.0/0.8	0.040	0	0	0.059	0	0	0	0	0	0
0.91	0	0	0	0	0	0	0.818	0	0.643	0
0.85	0	0	0	0	0	0	0.182	0.750	0.357	0
0.80	0.240	0	0	0	0	0	0	0	0	0
null	0	0	0	0	0	0	0	0	0	1.000
Het.	0.422	0.147	0	0.111	0.082	0	0.298	0.375	0.459	0
PO-1	25	25	23	17	23	9	11	4	14	12
1.00	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Het.	0	0	0	0	0	0	0	0	0	0
PO-2	25	25	23	17	23	9	11	4	14	12
1.30	0.040	0	0	0	0	0	0	0	0	0
1.00	0.960	1.000	1.000	1.000	0.870	1.000	1.000	1.000	1.000	1.000
0.97	0	0</								

PO-4	25	25	23	17	23	9	11	4	14	12
1.46	0	0	0	0	0	0	0	0	0	1.000
1.17	0	0	0	0	0	1.000	0	0	0	0
1.00	1.000	1.000	1.000	0.941	1.000	0	0	0	0	0
0.75	0	0	0	0.059	0	0	0	0	0	0
0.57/0.15	0	0	0	0	0	0	0	0.250	0	0
0.57	0	0	0	0	0	0	1.000	0.750	1.000	0
Het.	0	0	0	0.111	0	0	0	0.375	0	0
PGI	25	25	23	17	23	9	11	4	14	12
1.14	0	0	0	0.118	0	0	0	0	0.143	0
1.10	0	0	0	0	0	1.000	0	0	0.857	0.400
1.06	0	0	0	0	0	0	1.000	0	0	0
1.00	1.000	1.000	1.000	0.824	1.000	0	0	1.000	0	0.600
0.92	0	0	0	0.059	0	0	0	0	0	0
Het.	0	0	0	0.304	0	0	0	0	0.245	0.480
MDH-1	25	25	23	17	23	9	11	4	14	12
1.06	0	0	0	0.176	0	0.111	0.273	0.250	0.286	0
1.00	1.000	0.800	1.000	0.824	1.000	0.889	0	0.750	0	1.000
0.92	0	0	0	0	0	0	0.727	0	0.714	0
0.86	0	0.200	0	0	0	0	0	0	0	0
Het.	0	0.320	0	0.290	0	0.197	0.397	0.375	0.408	0
MDH-2	25	25	23	17	23	9	11	4	14	12
1.06	0	0	0	0	0	0.222	0.182	0	0	0
1.00	1.000	1.000	1.000	1.000	1.000	0.778	0.818	1.000	1.000	1.000
Het.	0	0	0	0	0	0.345	0.298	0	0	0
MDH-3	25	25	23	17	23	9	11	4	14	12
1.30	0	0	0	0	0.043	0	0	0	0	0
1.09	0	0	0.087	0	0	0.111	0	0	0	0.167
1.00	0.600	0.520	0.870	0.765	0.957	0.889	0.455	1.000	0.286	0.417
0.85	0.400	0.480	0.043	0.176	0	0	0.545	0	0.714	0.417
0.78	0	0	0	0.059	0	0	0	0	0	0
Het.	0.480	0.499	0.234	0.380	0.082	0.197	0.496	0	0.408	0.624
ACN	25	25	23	17	23	9	11	4	14	12
1.05	0	0.04	0	0	0	0	0	0	0	0
1.00	1.000	0.96	1.000	1.000	1.000	1.000	1.000	0.750	1.000	1.000
0.88	0	0	0	0	0	0	0	0.250	0	0
Het.	0	0.077	0	0	0	0	0	0.375	0	0
6-PGD	25	25	23	17	23	9	11	4	14	12
1.13	0	0	0	0	0	0.222	0	0	0	0
1.20	0	0	0	0	0	0	0	0	0.214	0.250
1.00	1.000	1.000	1.000	0.824	0.957	0.778	0	0	0.786	0.583
0.95	0	0	0	0.176	0.043	0	0	0	0	0.167
0.89	0	0	0	0	0	0	0.727	0.750	0	0
0.83	0	0	0	0	0	0	0.273	0.250	0	0
Het.	0	0	0	0.290	0.082	0.345	0.397	0.375	0.336	0.570
ME	25	25	5	17	23	9	11	4	14	12
1.10	0	0	0	0.118	0.043	0	0	0	0	0
1.06	0	0	0	0	0	0	0.727	0	0.286	0
1.00	0.960	1.000	0.800	0.882	0.957	1.000	0.273	1.000	0.714	1.000
0.90	0.040	0	0	0	0	0	0	0	0	0
0.88	0	0	0.200	0	0	0	0	0	0	0
Het.	0.077	0	0.32	0.208	0.082	0	0.397	0	0.408	0

GDH-1	25	25	23	17	5	9	11	4	14	12
1.15	0	0	0	0	0	0	0	0	0	0.917
1.05	0	0.120	0.261	0	0	0	0	0	0	0.083
1.00	0.680	0.720	0.478	0.412	0.800	0.333	0	0	0	0
0.90	0.240	0	0	0	0.200	0.667	0	0	0	0
0.89	0	0	0	0.412	0	0	0.727	1.000	0	0
0.74	0	0	0	0	0	0	0	0	0.786	0
0.50	0	0	0	0	0	0	0.091	0	0.214	0
null	0.080	0.160	0.261	0.176	0	0	0.182	0	0	0
Het.	0.474	0.442	0.635	0.630	0.320	0.444	0.430	0	0.336	0.152
GDH-2	25	25	23	17	5	9	11	4	14	12
1.16	0	0	0.087	0	0	0	0	0	0	0
1.07	0	0	0	0.176	0	0	0	0	0	0
1.00	1.000	0.920	0.913	0.824	0.800	1.000	0	1.000	0	0
0.80	0	0	0	0	0.200	0	0	0	0	0
0.64	0	0	0	0	0	0	0.455	0	0	0
0.57	0	0	0	0	0	0	0.545	0	0.786	0
null	0	0.08	0	0	0	0	0	0	0.214	1.000
Het.	0	0.147	0.159	0.290	0.320	0	0.496	0	0.336	0