Genetic structure of isolated selfers: *Chondrina clienta* (Westerlund, 1888) (Gastropoda: Stylommatophora) in Kraków-Częstochowa Upland

MAGDALENA SZAROWSKA, ANDRZEJ FALNIOWSKI & KRYSTYNA MAZAN-MAMCZARZ

Department of Malacology, Institute of Zoology, Jagiellonian University, 30-060 Kraków, Poland.
E-mail: SZAR@zuk.iz.uj.edu.pl

Abstract. The genetic structure of the lithophilous, xerothermic snail species *Chondrina clienta* (Westerlund, 1888) was studied, by means of cellulose acetate allozyme electrophoresis, in 14 peripherally isolated populations, at 12 localities in Kraków-Czestochowa Upland. 16 enzyme systems, coded by 25 loci, were assayed. In nine populations, all the studied loci were monomorphic, in the remaining five, one or two of the following loci were plymorphic: Aat-1, Aat-2, Est-1. Two of those populations were at Hardy-Weinberg equilibrium, in the other three populations statistically significant heterozygote deficiencies were observed. These are ascribable to selfing (suggested by the data from the literature on the common occurrence of the aphallic specimens) and/or inbreeding, while the observed very low polymorphism may be due to founder effects, bottlenecks, and probably very small evolutionary effective populations size of the studied populations. These would contribute to a very rapid heterozygosity decay. Gene flow estimates indicated the almost perfect isolation of each population. Thus, there is no source of new alleles to reestablish polymorphism in the studied populations; there also is a very little chance to restore a destroyed population by recolonization.

Kurzfassung. Untersucht wurde die genetische Struktur der lithophilen und xerothermen Schneckenart *Chondrina clienta* (Westerlund, 1888) mittels Celluloseacetat Allozym-Elektrophorese in 14 peripher isolierten Populationen im Bergland von Kraków-Czestochowa. 16 Enzymsysteme, kodiert durch 25 Loci, wurden geprüft. In neun Populationen erwiesen sich alle untersuchten Loci als monomorph, in den übrigen fünf waren einer oder zwei der folgenden Loci polymorph: Aat-1, Aat-2, est-1. Zwei dieser Populationen befanden sich im Hardy-Weinberg-Gleichgewicht, in den anderen drei Populationen wurden statistisch signifikante heterozygote Defizite festgestellt. Diese können auf Selbstbefruchtung (Literaturdaten über das allgemeine Auftreten von aphallischen Exemplaren weisen darauf hin) und/oder Inzucht zurückgeführt werden, während der festgestellte sehr geringe Polymorphismus durch Gründereffekte und wahrscheinlich zu geringe effektive Populationsgröße der untersuchten Populationen bedingt ist. Dies würde zu einem sehr raschen Verschwinden der Heterozygotie führen. Der geschätzte Genfluss zeigt die fast perfekte Isolation jeder Population an. Es existiert keine Quelle von neuen Allelen zur Wiederherstellung des Polymorphismus in den untersuchten Populationen, und die Chance, eine vernichtete Population durch Wiederbesiedlung wiederherzustellen, ist gering.

Key words. Gastropoda, *Chondrina clienta*, population, genetic structure, allozymes, electrophoresis, homozygote excess, selfing, inbreeding, gene flow, isolation.

Introduction

Chondrina clienta (Westerlund, 1888) is a small pulmonate snail, whose shell do not exceed 7 mm in height. This widely distributed and locally common species inhabits the mountains of the Central and Southeast Europe. It ranges from eastern Switzerland and southern Germany, through Slovakia, to the Balkans, Caucasus and Asia Minor; its isolated populations occur on the Baltic islands Öland and Gotland (RIEDEL 1988, BAUR 1988, BAUR et al. 1993). In Poland, it is common in the Tatra Mountains, Pieniny Mountains and Kraków-Częstochowa Upland. It is only on dry, calcareous rocks that this xerophilous and thermophilous, rock-

dwelling species can be found. It is nearly sedentary and feeds on lichens (BAUR 1988). The populations it forms are usually not rich in individuals.
In *Ch. clienta* (BAUR & KLEMM 1989) from five populations from Öland and one from Austria there was found complete lack of heterozygosity, explained mostly by self-fertilization, confirmed by high (52.2–99.1, mean 77.7%, depending on population) proportion of aphallic specimens (BAUR et al 1993). High proportion of aphallic specimens characterized also closely related *Ch. avenacea* from Switzerland (BAUR & CHEN 1993).
Kraków-Częstochowa Upland is situated at the northern border of the range of *Ch. clienta*, and is isolated from the main part of the range by a distance of about 75 km. Within the area, there are Jurassic limestones, forming small valleys or isolated rocks. Thus, the *Ch. clienta* populations of the Upland make a group of peripheral isolates at the border of the species range. Each of the populations inhabits its own "island" of calcareous rock and is isolated from the others. The aim of the study was to study at the genetic structure of such a subdivided, peripherally isolated population, looking for consequences of potential isolation and selfing. For this a cellulose acetate allozyme electrophoresis was applied.

Material and methods

Description of localities. Snails were picked from 14 demes, at 12 localities (Fig. 1) situated in Kraków-Częstochowa Upland. Kraków-Częstochowa Upland covers an area of 2,615 sq. km in the eastern part of Silesia-Kraków Upland. This is a plate of Upper Jurassic limestones. The Upland comprises four smaller units, the biggest of which is the northernmost unit, Częstochowa Upland covering almost a half of the total area. To the south, there go the following units: Olkusz Upland with its numerous buttes and brook valleys; Krzeszowice Foredeep, a 30 km long tectonic depression; Tenczyn Crest, a belt of hills, cut with numerous valleys of tributaries of the Vistula. All the material was collected in September–October 1999.
1. Skała Kmity (Kmita Rock), Rów Krzeszowicki (Krzeszowice Foredeep), 50°05.88'N, 19°49.20'E; a nature reserve, a steep, rocky hill covered with a mixed forest, 288 m a.s.l, at the border of the Rudawa River valley in its gate part; on dry rocks on top of the hill.
2–5. Ojcowski Park Narodowy (Ojców National Park), the valley of the Prądnik River, Wyżyna Olkuska (Olkusz Upland): 2. Brama Krakowska (Gate of Kraków), 50°11.40'N, 19°50.28'E; on the rocks of the „gate"; 3. Skała Krukowskiego (Krukowski Rock), 50°11.89'N, 19°49.78'E; 4. 50°13.45'N, 19°49.77'E, on a rock by the main road, at the Jaskinia Ciemna (Dark Cave) ticket office; 5. Pieskowa Skała, Maczuga Herkulesa (Hercules Mace Rock), 50°14.56'N, 19°47.03'E, on a rock by the main road.
6–8. Rezerwat Dolina Bolechowicka (Bolechowice Valley Nature Reserve), Wyżyna Olkuska (Olkusz Upland), 50°09.15'N, 19°47.34'E, rocks at the mouth of the valley: 6 and 8 on two rocks on the left-hand side of the brook, about 40 m distant from each other, connected by smaller rocks; 7 on a rock on the right-hand side of the brook, opposite to 8, about 40 m to the west.
9. Dolina Kobylańska (Kobylany Valley), Wyżyna Olkuska (Olkusz Upland), 50°09.37'N, 19°45.38'E, a rock at the mouth of the valley.
10. Dolina Będkowska (Będkowice Valley), Wyżyna Olkuska (Olkusz Upland), 50°09.92'N, 19°44.44'E, a rock on the left-hand side of the Będkówka stream, about the middle of the valley.
11. Olsztyn, Wyżyna Częstochowska (Częstochowa Upland), 50°44.94'N, 19°16.62'E, at the ruins of the medieval castle, on a small rock in a meadow.
12. Rezerwat Góra Zborów (Congregation Mountain Nature Reserve), Wyżyna Częstochowska (Częstochowa Upland), 50°34.46'N, 19°31.48'E; a rocky hill, the highest one of the Kroczyckie Skałki Belt, 463 m a.s.l., on a small rock in a beech forest.
13. Ogrodzieniec, Wyżyna Częstochowska (Częstochowa Upland), at the ruins of the medieval castle, 50°27.41'N, 19°33.32'E; on a small rock in a beech forest.
14. Rezerwat Wąwóz Mnikowski (Mników Gorge Nature Reserve), Grzbiet Tenczyński (Tenczyn Crest), 50°04.09'N, 19°42.36'E, a rock at the entrance to the reserve, on the left-hand side of the Sanka river.

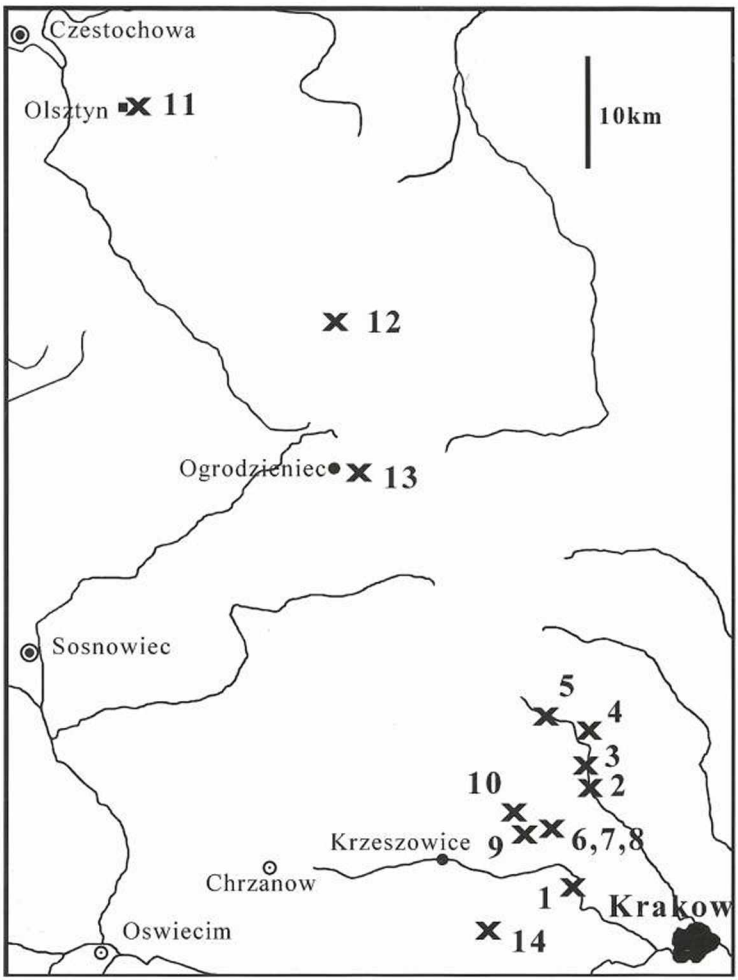

Fig. 1. Map of studied localities. Bar indicates 10 km; for locality names, see the text.

Collection and electrophoretic techniques. Snails were collected by hand from rocks. Attention was paid not to mix specimens from more than one deme. To avoid this, thus to avoid Wahlund's effect, they were always collected from a surface of a few square metres. In the laboratory, the gastropods were immediately frozen in a deep freezer at -80 °C and kept there until usage. Individuals for electrophoresis were homogenized in a glass homogenizer, in 50 µl of homogenizing solution. The homogenates were centrifuged at 11.000 RPM for 10 min. and used immediately for cellulose acetate electrophoresis following the protocol of RICHARDSON et al (1986). The cellogel strips were from MALTA, Italy; the other chemicals from SIGMA, USA. The enzyme names and E.C. codes are after MURPHY et al. (1996).

Numerical techniques. All data were analyzed with GENEPOP (RAYMOND & ROUSSET 1995), to compute exact tests for HWE (GUO & THOMPSON 1992, ROUSSET & RAYMOND 1995) and f (= F_{IS} in WRIGHT 1978) for each polymorphic population and for each polymorphic locus, with exact probabilities. The proportion of progeny produced by self-fertilization was estimated for each polymorphic loci in each population after formula given by HEDRICK (1983):

Tab. 1. Genetic variability at 25 loci in all studied populations; s.e. in parentheses, *unbiased estimate.

Population	Sample size	Percentage of loci polymorphic	Mean heterozygosity Direct count	HWE expected*
1	24	0.0	0.000 (0.000)	0.000 (0.000)
2	22	0.0	0.000 (0.000)	0.000 (0.000)
3	26	0.0	0.000 (0.000)	0.000 (0.000)
4	24	0.0	0.000 (0.000)	0.000 (0.000)
5	35	4.0	0.007 (0.007)	0.017 (0.017)
6	21	0.0	0.000 (0.000)	0.000 (0.000)
7	23	0.0	0.000 (0.000)	0.000 (0.000)
8	13	0.0	0.000 (0.000)	0.000 (0.000)
9	24	0.0	0.000 (0.000)	0.000 (0.000)
10	30	8.0	0.008 (0.008)	0.022 (0.019)
11	24	4.0	0.005 (0.005)	0.005 (0.005)
12	23	4.0	0.003 (0.003)	0.016 (0.016)
14	24	4.0	0.015 (0.015)	0.020 (0.020)
mean	24	1.8	0.003 (0.003)	0.006 (0.006)

S = (4pq-2h)/(4pq-h), where p and q are allele frequencies, and h is the proportion of heterozygous individuals for a given locus. F-statistics were computed over all populations of *Ch. clienta,* with WEIR'S FSTAT (WEIR 1990, GOUDET 1995), following the notation and computational procedure introduced by WEIR & COCKERHAM (1984) and WEIR (1990), with confidence intervals estimated with jackknife and bootstrapping techniques. FSTAT was also applied to calculate θ (= F_{ST} in WRIGHT 1978) and Nm values for all possible pairs of populations. Mantel tests were performed with NTSYSpc (ROHLF 1998). Nm estimate was also calculated with GENEPOP, applying the private allele technique (SLATKIN 1985). Composite linkage disequilibrium coefficient Δij (the best disequilibrium coefficient for analysis of field data, assuming neither random mating nor gametes' frequencies: BLACK & KRAFSUR 1985), corrected correlation coefficient Rij (BLACK & KRAFSUR 1985), and D-statistics of OHTA (1982) were computed with LINKDOS (GARNIER-GERE & DILLMANN 1992). Mean heterozygosities and genetic distances were computed with BIOSYS-1 (SWOFFORD & SELANDER 1981).

Results

16 enzyme systems, coded by 25 loci, were assayed and gave always scorable and interpretable results. These were: Alcohol Dehydrogenase (ADH: EC 1.1.1.1); Alkaline Phosphatase (ALP: EC 3.1.3.1); Aspartate Aminotransferase (AAT: EC 2.6.1.1, two loci); Esterases (EST: non-specific, four loci); Fumarate Hydratase (FUMH: EC 4.2.1.2); Glucose-6-Phosphate Dehydrogenase (G6PDH: EC 1.1.1.49); Glucose-6-Phosphate Isomerase (GPI: EC 5.3.1.9); Glycerol-3-Phosphate Dehydrogenase (G3PDH: EC 1.1.1.8); 3-Hydroxybutyrate Dehydrogenase (HBDH: EC 1.1.1.30, three loci); Isocitrate Dehydrogenase (IDH: EC 1.1.1.42, two loci); Malate Dehydrogenase (MDH: EC 1.1.1.37, two loci); Mannose-6-

Tab. 2. Cavalli-Sforza & Edwards arc genetic distance (below diagonal) and Nei unbiased genetic distance (above diagonal) computed for each pair of populations; distances equalling 0 in italics.

	1	2	3	4	5	6	7	8	9	10	11	12	14.
1	*.***	*0.000*	*0.000*	*0.000*	0.019	*0.000*	*0.000*	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
2	*0.000*	*.***	*0.000*	*0.000*	0.019	*0.000*	*0.000*	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
3	*0.000*	*0.000*	*.***	*0.000*	0.019	*0.000*	*0.000*	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
4	*0.000*	*0.000*	*0.000*	*.***	0.019	*0.000*	*0.000*	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
5	0.124	0.124	0.124	0.124	*.***	0.019	0.019	0.019	0.019	0.025	0.019	0.042	0.027
6	*0.000*	*0.000*	*0.000*	*0.000*	0.124	*.***	*0.000*	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
7	*0.000*	*0.000*	*0.000*	*0.000*	0.124	*0.000*	*.***	*0.000*	*0.000*	0.005	*0.000*	0.022	0.007
8	*0.000*	*0.000*	*0.000*	*0.000*	0.124	*0.000*	*0.000*	*.***	*0.000*	0.005	*0.000*	0.022	0.007
9	*0.000*	*0.000*	*0.000*	*0.000*	0.124	*0.000*	*0.000*	*0.000*	*.***	0.005	*0.000*	0.022	0.007
10	0.086	0.086	0.086	0.086	0.151	0.086	0.086	0.086	0.086	*.***	0.004	0.005	*0.000*
11	0.032	0.032	0.032	0.032	0.128	0.032	0.032	0.032	0.032	0.056	*.***	0.018	0.005
12	0.132	0.132	0.132	0.132	0.181	0.132	0.132	0.132	0.132	0.054	0.100	*.***	0.003
14	0.092	0.092	0.092	0.092	0.155	0.092	0.092	0.092	0.092	0.025	0.060	0.040	*.***

Phosphate Isomerase (MPI: EC 5.3.1.8); Phosphoglucomutase (PGM: EC 5.4.2.2, two loci); Phosphogluconate Dehydrogenase (PGDH: EC 1.1.1.44); Purine-Nucleoside Phosphorylase (PNP: EC 2.4.2.1); Triose-Phosphate Isomerase (TPI: EC 5.3.1.1).

In population 13, enzyme activity was observed in only one specimen, thus the population was excluded from analysis. In most of the populations (all except 5, 10, 11, 12 and 14), all the 25 loci were monomorphic in all individuals (Table 1). Genetic polymorphism was observed in Aat-1 in population 5, in Est-1 in populations 11, 12 and 14, and in Aat-2 and Est-1 in population 10. Thus, there were 0–8%, mean 1.8%, of polymorphic loci, depending on population. Mean observed heterozygosity varied between 0.000 and 0.015, mean 0.003; mean expected heterozygosity was 0.000–0.022, mean 0.006 (Table 1). Cavalli-Sforza & Edwards arc genetic distance between populations (Table 2) ranged from 0.000 to 0,181 (the latter between populations 5 and 12). Computed for the five polymorphic populations only, it was not statistically significantly associated (Mantel test: p=0.1415) with geographic distances between the populations. Unbiased Nei genetic distance (Table 2) ranged from 0.000 to 0.042 (the highest value was between 5 and 12), and for five polymorphic populations was not significantly associated (Mantel test: p=0.4190) with geographic distances between the populations. All the subsequent analyses were restricted to the five polymorphic populations: 5, 10, 11, 12 and 14.

Linkage disequilibrium computed with Fisher's technique did not indicate a statistically significant disequilibrium between any pair of the polymorphic loci. In population 14, for Aat-2-Est-1, composite disequilibrium Δ_{ij} equalled ± 0.04368, the corrected correlation coefficient R_{ij} was ± 0.28501, $\chi^2=2.44$, $p \leq 0.1185$. For all populations the values were as follows: for Aat-2-Est-1: $\Delta_{ij}=\pm 0.00518$, $R_{ij}=\pm 0.13305$, $\chi^2=5.54$, $p \leq 0.0186$; for Aat-1-Est-1: $\Delta_{ij}=\pm 0.01966$, $R_{ij}=\pm 0.12382$, $\chi^2=4.80$, $p \leq 0.0285$. Ohta's D-statistics was computed for each pair of loci. $D'_{IS}{}^2$ was always higher than $D'_{ST}{}^2$, and $D_{ST}{}^2$ was always higher than $D_{IS}{}^2$. Comparisons between the expected and observed mean heterozygosity mentioned above suggest a heterozygote deficit. The GENEPOP exact multilocus tests detected a highly significant homozygote excess in populations 5, 10 and 12, and an almost significant homozygote excess in the remaining two populations (Table 3). The multipopulation tests showed an almost not significant deficit at Aat-2. In fact, Aat-2 was polymorphic only in population 10, and the locus was there practically fixed at one allele. A similar, highly significant heterozygote deficit we detected at Aat-1 in population 5. At Est-1 a highly significant heterozygote deficit was detected in two populations (10 and 12) and in the other two Hardy-Weinberg equilibrium was postulated (Table 3). Similar picture was presented by the values of f. Estimates of percent of progeny produced by self-fertilization varied between populations, from 0 to 100% (Table 3).

For the polymorphic loci, we computed F-statistics over the five polymorphic populations (Table 4). To numerical resampling we applied the random model of WEIR (1990). The confidence intervals for the coancestry coefficient θ were similar in width to, although contained much lower values than, the confidence intervals for either the intrapopulation inbreeding coefficient f or overall inbreeding coefficient F (Table 4). This pointed to f and θ as equally significant sources of variation. This was also indicated by the mean values and standard deviations computed by jackknifing over loci. It must be stressed that the values above one, computed for Aat-1, reflect no more than the nature of the jackknife estimate and not a biological reality. The mean values over populations computed for particular loci indicate that the intervals of the coancestry coefficient θ do not overlap between loci; however, we did not calculate the estimates for the same populations. All of the polymorphic populations except one were polymorphic at only one locus. For Aat-1 and Est-1, F, θ and f were statistically highly significant. For the former locus, θ and f were nearly equal, whereas for the latter f was somewhat higher than θ (Table 4).

The value of θ over all loci was 0.487, and the estimate of migrant number computed from it equaled 0.26. Private allele technique resulted in p(1)=0.3595, and number of migrants after correction for size Nm=0.064. Pairwise coancestry coefficient and the corresponding Nm values for each pair of polymorphic populations were computed also (Table 5). Pairwise θ was reaching 0.7024 between populations 5 and 12. It was not significantly associated with the geographic distance between the populations (Mantel test: p=0.3723). Paiwise Nm values were low, from 0.106 to 1.067, and not significantly associated with geographic distance between populations (Mantel test: p=0.3633).

Tab. 3. Exact probabilities: p, Pml and Pmp, calculated with Genepop by complete enumeration, assuming H_0=HWE (Hardy and Weinberg equilibrium) and H_1= homocygote deficit (for H_1=heterozygote excess each test gave not significant levels of probability). p – for a given locus and population, Pml – multi-locus (by population) and Pmp – multi-population (by locus), standard errors in parentheses. f – values of f (after WEIR & COCKERHAM 1984), h% – percent of heterozygotes, S% – percent of progeny produced by self-fertilization (after HEDRICK 1983), for each polymorphic locus and population.

pop.	locus	D?	p	f	h%	S%	Pml	Pmp
5.	Aat-1	D	0.0005	+0.611	17.14%	75.2%	0.0000(±0.0000)	0.0000(±0.0000)
10.	Aat-2	D	0.0169	+1.000	0.0%	100.0%	0.0000(±0.0000)	0.0168(±0.0004)
10.	Est-1	D	0.0021	+0.581	25.0%	63.2%	0.0000(±0.0000)	0.0002(±0.0000)
11.	Est-1	HWE	1.0000	-0.045	12.5%	0.0%	0.0635(±0.0008)	0.0002(±0.0000)
12.	Est-1	D	0.0005	+0.783	8.7%	87.2%	0.0000(±0.0000)	0.0002(±0.0000)
14.	Est-1	HWE	0.1936	+0.258	37.5%	38.4%	0.0443(±0.0009)	0.0002(±0.0000)

Tab. 4. F-statistics over all populations, for all polymorphic loci (excluding not significant values for Aat-2), computed with FSTAT; [probability that f or F or θ is not >0] <0.00007, except for the values given *, whose probability is within [0.0000...0.00067]; Mean o-pop: mean and standard deviation (in brackets) computed with jackknifing over populations; Mean o-loci: mean and s.d. (in brackets) computed over loci; Boot o-loci – bootstrapping over loci, 99% confidence intervals.

	Allele	F	θ	f
Aat-1	a+b	0.869	0.661	0.614*
Mean o-pop		1.569 (±0.694)	1.200(±0.526)	1.106* (±0.491)
Est-1	a+b	0.674	0.373	0.480
Mean o-pop		0.695 (±0.163)	0.384(±0.205)	0.488 (±0.141)
Over all loci		0.762	0.487	0.536
Mean o-loci		0.733 (±0.133)	0.459(±0.188)	0.465 (±0.123)
Boot o-loci		[0.674 1.000]	[-0.004 0.661]	[0.480 1.000]

Tab. 5. Pairwise θ (below diagonal) and Nm (above diagonal) between the polymorphic populations.

	5.	10.	11.	12.	14.
5.	*.***	0.206	0.167	0.106	0.180
10.	0.5486	*.***	1.067	0.970	x.xxx
11.	0.5994	0.1898	*.***	0.143	0.588
12.	0.7024	0.2049	0.6358	*.***	1.483
14.	0.5816	-0.0162	0.2982	0.1443	*.***

Discussion

Land pulmonates are usually genetically polymorphic, but sometimes they are not (CLARKE et al. 1978, FOLTZ et al. 1982, HILLIS et al. 1987, HILLIS 1989). BAUR & KLEMM (1989) found no intrapopulation polymorphism in *Ch. clienta*, and BOATO (1988) found low or medium genetic variation, heterozygote deficiencies and restricted gene flow in *Solatopupa*, belonging also to Chondrinidae. The total lack of allozyme polymorphism in eight of the studied populations resembles the data of BAUR & KLEMM (1989). It may be due to founder events, or may suggest that these rather small and isolated populations went through a bottleneck. Or else, it simply may be a result of those gastropods' generation time not exceeding three years (BAUR 1988), this coupled with the extremely small size of a deme. It seems that in *Ch. clienta* evolutionary effective population size is very low, perhaps a few dozen individuals. In such populations, the evolutionary processes, like polymorphism decay, are relatively very rapid. On the other hand, there is too little time for new mutations, thus all the new alleles that may appear are the ones brought in by immigrants. In such populations, when migration is very limited (see below), almost all the loci will be fixed at one allele in a relatively short time. Compared with the literature, even in the five polymorphic populations only, the proportion of polymorphic loci was very low. Those values, however, are not exceptionally low for terrestrial pulmonates (HILLIS 1979, HILLIS et al. 1987). The mean expected heterozygosity in all the five polymorphic populations is below the average for pulmonates (see FALNIOWSKI et al. 1996 for references), and far below the 0.15 average for mollusks (NEVO et al. 1984). The mean observed values are lower than the expected ones, pointing, in general, to a heterozygote deficit. The interpopulation variation, reflected in both unbiased Nei genetic distance and Cavalli-Sforza & Edwards arc genetic distance, is very small for even intraspecific comparisons (FALNIOWSKI et al. 1996 for references; similar distances were found for the species between the Austrian and Swedish populations: BAUR & KLEMM 1989). They were not associated with geographic distances, thus the island model, not the stepping-stone model, seems to apply to the studied case. It is in agreement with what one may expect concerning the isolated, small habitats of rocks. This island model we had expected to concern *Bythinella* populations inhabiting isolated springs but it was not confirmed in practice (FALNIOWSKI et al. 1998).

In the studied *Chondrina* populations, Δ_{ij} and R_{ij} were low but statistically significant for Aat-2-Est-1 and Aat-1-Est-1. Ohta's D-statistics indicated very high values of $D'_{IS}{}^2$ and $D_{IT}{}^2$ for Aat-1-Est-1. All the above may reflect gametic disequilibrium, caused by selfing (JARNE et al. 1993). $D'_{IS}{}^2$ is the expected variance of the correlation of two alleles at two loci in one gamete in a subpopulation, which is relative to that in the total population. It is the variance of the correlation of the two alleles at two loci on one chromosome in a population, which is relative to that in the total population (OHTA 1982). The general pattern, in which the values of $D'_{IS}{}^2$ are always higher than those of $D'_{ST}{}^2$, and the values of $D_{ST}{}^2$ are always higher than those of $D_{IS}{}^2$, points to this being a nonsystematic disequilibrium (OHTA 1982, BLACK & KRAFSUR 1985): the main source of the disequilibrium is limited migration, certainly not the natural epistatic selection that favours gametes with the same allele combinations in every population (OHTA 1982). We must also exclude the strong epistatic selection in some of the populations only.

The multipopulation tests for each of the loci have confirmed the occurrence of heterozygote deficit in the polymorphic populations. For Est-1, the deficit was significant in two populations, and in the other two it was not. Such a deficit may be due to the common occurrence, in the considered populations, of either selfing or inbreeding, or some other disturbing forces (e.g. WEIR 1990). One of the latter is Wahlund's effect, which is due to the spatial differentiation of populations and may appear in even a very restricted area (GRANT & UTTER 1988, DAY 1990, JOHNSON et al. 1993). However, we took our samples from only a few sq. m. of area, so Wahlund's effect seems improbable. In fact, it is not very probable for all the loci, and heterozygote deficiencies in three populations are congruent with the complete lack of polymorphism in the other eight populations. The almost sedentary mode of life, hermaphroditism, and extremely small evolutionary effective population size, are the prerequisites for selfing and/or inbreeding. Inbreeding is an evidently overexploited explanation of heterozygote deficits in gastropods (FALNIOWSKI et al. 1999), yet it may be the main factor causing heterozygote deficit in *Ch. clienta*.

The high values of θ confirm selfing/inbreeding: if one of these occurred, the coancestry coefficient would assume high values even between very close populations (HILLIS 1989) like between populations 5 and 10 of the studied *Ch. clienta*. The results F-statistics also suggest selfing and/or inbreeding as the main source of homozygote excess. On the other hand, the complete lack of polymorphism in eight populations, coupled with the very low level of polymorphism in the other ones, seem to suggest some contribution of codominant selection to the observed pattern. Such selection cannot, however, be strong, according to the results of D-statistics. If the evolutionary effective size of a population is small, like it seems to be in *Ch. clienta,* even an extremely small difference in fitness between alleles may result in the fixation of the population at one allele in a relatively short time.

The data of BAUR & CHEN (1993) and BAUR et al. (1993) strongly indicate selfing as the main source of the observed homozygote excess. Although all the pulmonates and Heterobranchia are hermaphroditous, there is no general pattern of the mating system in those snails (DUNCAN 1975, FOLTZ et al. 1982, GERAERTS & JOOSSE 1984, TOMPA 1984, JARNE et al. 1993, FALNIOWSKI et al. 2000). Most species studied so far avoids selfing as long as possible, but in some species selfing seems common (e.g. FOLTZ et al. 1982), and in some other selfing is the main mode of reproduction (SELANDER et al. 1974, HILLIS et al. 1987, BAUR 1989, BAUR & KLEMM 1989, HILLIS 1989); *Ch. clienta* seems one more example of nearly sedentary snail forming small populations inhabiting isolated microhabitats, for which selfing promotes colonization and recovering after bottle-necks.

The value of the coancestry coefficient in *Ch. clienta* is as high as in *Bythinella* (FALNIOWSKI et al. 1999), although in *Solatopupa* the values were similar or even somewhat higher (BOATO 1988). Of course, it has been calculated for only five polymorphic populations, and, what is more important, for one or two loci in each population. Thus, it may be not much reliable. However, there is no other way to calculate this coefficient and we have to interpret it as it is. This coefficient also suggests the island, not the stepping-stone, model for the studied populations. Similar criticism must concern the gene flow estimates, either these calculated from θ or those calculated from private alleles. The latter value is lower than the one calculated from the analytic theory, but the detected number of the polymorphic loci was extremely low and private alleles are easily overlooked (e.g. LESSIOS & WEINBERG 1993, FALNIOWSKI et al. 1999). However, the value was not less than one fourth the Nm value calculated from θ. The estimates yielded by either techniques indicate a very low level of gene flow between the polymorphic populations of *Chondrina*.

All the techniques of indirect estimates of gene flow are subject to much criticism (e.g. AVISE 2000) so one should be more than careful when interpreting the results. However, there is no other technique available in most cases like *Chondrina*. Last but not least, the estimates are congruent with all the other results and data concerning the population genetic structure of the subdivided population of *Chondrina* and the biology of this snail, whose annual dispersal equalled 86 cm, and never exceeded 343 cm (BAUR 1988), and associated exclusively with the calcareous rocks. According to BAUR (1988, BAUR & KLEMM 1989), in Öland colonization is not possible by subsequent active dispersal, but only accidental transport by winds, water, birds and humans. In fact, his opinion about the origin of the Öland

populations, as relicts from warmer periods, seems doubtful in the light of the obligatory association of the species with calcareous rocks, absent in the wide discontinuity of the species range. In our opinion, the Öland populations are introduced by man, perhaps with the stones from the Mediterranean coasts of the Balkans or Asia Minor, used as balast for the sailing ships. This may also explain the total lack of allozymic differentiation, either intra- or interpopulation, observed by BAUR & KLEMM (1989). It seems that there practically are no immigrants in the studied Polish populations. Calcareous rocks are like islands, each one of them harbouring a population which is isolated from the others and unique. From the point of view of conservation biology each of the populations may easily be destroyed, but would rather not reappear due to recolonization.

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